**University of Lille**

**Doctoral school Biology-Health of Lille**

# Study of maternal-mediated mechanisms in the epigenetic programming induced by maternal stress: Transgenerational transmission and oxytocin

**Ryma BENLAKEHAL**

Defended on September 23rd, 2024

For the grade of **Doctor in Neurosciences** at **Lille University (France)**

(14h, Amphi B, at Lilliad Cité Scientifique)

**Members of the jury**

Prof. Stefania Maccari (University of Lille, France).....President of the jury

Prof. Paula Brunton (University of Edinburgh, UK).....Thesis reporter

Prof. Ferdinando Nicoletti (Sapienza University of Rome, Italy).....Thesis reporter

Prof. Mohamed Kabbaj (FSU College of Medecine, USA).....Thesis examiner

Dr. Stephanie Olivier-Van Stichelen (Medical College of Wisconsin, USA).....Thesis examiner

Dr. Sébastien Bouret (University of Lille, France).....Thesis examiner

Prof. Sara Morley-Fletcher (University of Lille, France).....Thesis supervisor

Dr. Anne Harduin-Lepers (University of Lille, France).....Thesis co-supervisor

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# Abstract

Adverse environmental factors play a major role in health and disease development, among these factors is stress, especially when it occurs during critical periods, such as the perinatal period (prenatal and postnatal), it increases maternal glucocorticoids and reduces maternal behavior, leading to maladaptive programming in the offspring. Using the perinatal stress (PRS) model in rats, my PhD project aimed to investigate the transmission of PRS-induced deficits through the maternal line to subsequent generations *via* intergenerational and transgenerational inheritance, and to uncover the mechanisms of multigenerational transmission *via* activation of the oxytocinergic system through *postpartum* treatment of the stressed mother. This study is the first to examine the transmission as well as the reversal of PRS deficits through multiple generations. Thus, we explored the corrective effect of enhancing maternal behavior through oxytocinergic activation using an oxytocin analog (carbetocin: CBT; injected intraperitoneally and intranasally) or an alternative approach via the probiotic *Limosilactobacillus reuteri* (administered in drinking water) which is known to display oxytocinergic activity. Our findings revealed that gestational stress in F0 dams reduced maternal care over three generations until F2 dams. Additionally, stressed dams showed a disturbed stress/anti-stress balance and Hypothalamic-Pituitary-Adrenal (HPA) axis dysfunction, characterized by increased corticosterone levels (CORT) and reduced oxytocin (OT) in the plasma. Moreover, stressed mothers exhibited reduced OT levels and its receptor OTR in the hypothalamus, alongside increased BDNF (Brain-Derived Neurotrophic Factor) and its isoforms. These maternal changes led to disturbances in the offspring, including impaired risk-taking behavior in the Elevated-Plus Maze (EPM) and an imbalance in the HPA axis, persisting transgenerationally up to the F3 offspring. This was accompanied by neurochemical changes in the hippocampus of the PRS offspring, including increased BDNF and reduced MR (mineralocorticoid receptors), GR (glucocorticoid receptors), and mGluR2/3 (metabotropic glutamate receptors 2 and 3) up to the F2 generation. Overall, robust correlations were highlighted between early-life exposure in mothers and changes in offspring over multiple generations. Remarkably, both intraperitoneal and intranasal CBT successfully increased peripheral OT levels in F0 treated dams with consequent enhancement of maternal care which in turn rescued all the behavioral and neurochemical deficits studied in the PRS offspring which persisted up to the F2 generation. We also provided groundbreaking evidence of *L. reuteri* action on maternal behavior in the stressed dams with associated elevation of OT levels in both plasma and hypothalamus and normalization of hypothalamic BDNF levels to those of control unstressed dams. Again, the reversal of maternal behavior deficit induced by *L. reuteri* benefited the PRS offspring.

This study underscored the importance of maternal care and the *postpartum* period, and oxytocinergic activation appears to exert beneficial effects through a mechanism involving the stress/anti-stress balance and the OT/BDNF interplay. Together, these findings suggest potential therapeutic strategies for mitigating the effects of early-life stress and improving the health of the mother-infant dyad.



**Keywords**: Maternal care, Early-life stress, Oxytocin, *Limosilactobacillus reuteri* (probiotic), BDNF, Epigenetics, Intergenerational transmission, Transgenerational transmission.

**Université de Lille**

**École Doctorale Biologie-Santé de Lille**

# Étude des mécanismes à médiation maternelle dans la programmation épigénétique induite par le stress maternel : Transmission transgénérationnelle et ocytocine

**Ryma BENLAKEHAL**

Soutenue le 23 septembre 2024

Pour le grade de **Docteur en Neurosciences** à l'Université de Lille, France

**Membres du jury**

Prof. Stefania Maccari (Université de Lille, France)..... .....Présidente du jury

Prof. Paula Brunton (Université d'Édimbourg, Royaume-Uni).....Rapportrice

Prof. Ferdinando Nicoletti (Université Sapienza de Rome, Italie).....Rapporteur

Prof. Mohamed Kabbaj (FSU Collège de Médecine, États-Unis).....Examinateur

Dr. Stephanie Olivier-Van Stichelen (École de Médecine du Wisconsin, États-Unis)..... Examinatrice

Dr. Sébastien Bouret (Université de Lille, France).....Examinateur

Prof. Sara Morley-Fletcher (Université de Lille, France).....Directrice de thèse

Dr. Anne Harduin-Lepers (Université de Lille, France).....Co-directrice de thèse

# Résumé

Les facteurs environnementaux défavorables jouent un rôle majeur dans la santé et le développement des maladies, parmi ces facteurs figure le stress, surtout lorsqu'il survient durant des périodes critiques telles que la période périnatale (prénatale et postnatale). Le stress augmente les glucocorticoïdes maternels et réduit le comportement maternel, entraînant une programmation inadaptée chez la progéniture. En utilisant le modèle de stress périnatale (PRS) chez le rat, mon projet de doctorat vise à étudier la transmission des déficits induits par le PRS à travers la lignée maternelle vers les générations suivantes via l'hérédité intergénérationnelle et transgénérationnelle, et à explorer les mécanismes de la transmission sur plusieur générations par l'activation du système ocytocinergique grâce au traitement *postpartum* des mères stressées. Cette étude est la première à examiner la transmission ainsi que la correction des déficits induits par le PRS à travers plusieurs générations. Ainsi, nous avons exploré l'effet correcteur de l'amélioration du comportement maternel par l'activation ocytocinergique en utilisant un analogue de l'ocytocine (carbétocine : CBT ; injecté par voie intrapéritonéale ou intranasale) ou une approche alternative via le probiotique *Limosilactobacillus reuteri (L. reuteri)*, connu pour son activité ocytocinergique. Nos résultats ont révélé que le stress gestationnel chez les mères F0 réduisait les soins maternels sur trois générations jusqu'aux mères F2. De plus, les mères stressées présentaient un déséquilibre du stress/anti-stress et un dysfonctionnement de l'axe Hypothalamo-Hypophyso-Surrénalien (HPA), caractérisé par une augmentation des niveaux de corticostérone (CORT) et une réduction de l'ocytocine (OT) dans le plasma. Par ailleurs, les mères stressées présentaient des niveaux réduits d'OT et de son récepteur OTR dans l'hypothalamus, ainsi qu'une augmentation du BDNF (Brain-Derived Neurotrophic Factor) et de ses isoformes. Ces changements maternels ont conduit à des perturbations chez la progéniture, y compris un comportement de prise de risque altéré dans le labyrinthe en croix surélevé (EPM) et un déséquilibre de l'axe HPA, persistant de manière transgénérationnelle jusqu'à la progéniture F3. Cela s'accompagnait de changements neurochimiques dans l'hippocampe de la progéniture PRS, y compris une augmentation du BDNF et une réduction des récepteurs des minéralocorticoïdes (MR), des récepteurs des glucocorticoïdes (GR) et des récepteurs métabotropiques du glutamate 2 et 3 (mGluR2/3) jusqu'à la génération F2. Globalement, des corrélations robustes ont été mises en évidence entre l'exposition précoce chez les mères et les changements chez la progéniture sur plusieurs générations. Remarquablement, le CBT IP et IN ont réussi à augmenter les niveaux périphériques d'OT chez les mères F0 traitées, avec une amélioration conséquente des soins maternels qui a à son tour corrigé les déficits comportementaux et neurochimiques observés chez la progéniture PRS qui persistaient jusqu'à la génération F2. Nous avons également démontré l'action de *L. reuteri* sur l'augmentation du comportement maternel chez les mères stressées associée à une augmentation d'OT dans le plasma et l'hypothalamus, et une normalisation des niveaux hypothalamiques de BDNF à ceux des mères controles. Là encore, la réversion du déficit de comportement maternel induit par *L. reuteri* a bénéficié à la progéniture PRS.

Cette étude a souligné l'importance des soins maternels et de la période *postpartum*, et l'activation ocytocinergique qui exerce des effets bénéfiques via un mécanisme impliquant l'équilibre stress/anti-stress et l'interaction OT/BDNF. Ensemble, ces résultats suggèrent des stratégies thérapeutiques potentielles pour atténuer les effets du stress précoce et améliorer la santé de la dyade mère-enfant.

**Mots-clés** : Soins maternels, Stress précoce, Ocytocine, *Limosilactobacillus reuteri* (probiotique), BDNF, Épigénétique, Transmission intergénérationnelle, Transmission transgénérationnelle.

# Preamble

Over the years, the field of neuroscience has witnessed remarkable evolution, driven by groundbreaking discoveries and technological advancements. This dynamic discipline has expanded our understanding of the brain and its intricate processes, shedding light on both normal and pathological conditions. During my PhD, I have focused on stress programming and the transmission of both its deficits and corrections.

This dissertation presents the results of my efforts to explore these less-charted territories, employing the resources and methodologies accessible to me. By concentrating on the potential for correcting stress-induced neurobiological changes, my work seeks to contribute to a more comprehensive understanding of how early life stress affects the developmental trajectory of health. This approach not only aligns with the existing body of literature that explores the mechanisms of stress transmission but also introduces new perspectives on mitigating its long-term impacts.

Central to my research is the exploration of the oxytocin system and its mechanisms in the long-term programming of maternal stress within the context of the stress/anti-stress balance. Moreover, I explored oxytocin as a treatment during the *postpartum* period to mitigate deficits related to gestational stress and its impact on the mother and the descendants, as well as its persistence across generations. I have investigated maternal oxytocin's effects on behavior, hormonal balance, and brain biomarkers, aiming to uncover therapeutic strategies that can enhance resilience and promote well-being in individuals affected by early life stress. I have focused on the mother-pup dyad as well as on the mother and the offspring separately.

From this work, two main chapters emerged. In the **first chapter** (with *a submitted article* and *an article in preparation*), my aim was to highlight the pivotal role of maternal behavior in the intergenerational transmission of gestational stress from F0 dams up to F2 offspring, and the reversal of PRS-related deficits *via postpartum* carbetocin treatments solely in F0 dams. The second segment addressed the transgenerational transmission of PRS-related deficits up to F3 males, examining the persistent effects of intraperitoneal and intranasal carbetocin treatments initiated in *postpartum* F0 dams. In the **second chapter**, (with *a submitted article* and *an article in preparation*), I aimed to uncover the surprisingly beneficial effect of *postpartum* probiotic *L. reuteri* in rescuing deficits caused by gestational stress. First, I explored the impact of gestational stress on mothers and the corrective effect of *postpartum L. reuteri* treatment on behavioral, neurochemical, and endocrinological parameters. Then, I focused on the offspring, examining the consequences of PRS and the ability of maternal *L. reuteri* treatment to mitigate maladaptive programming in behavioral, endocrinological, and neurochemical parameters.

Through this research, I aspired to offer insights into existing gaps and provide a foundation for future studies aimed at developing therapeutic interventions in the field of early-life

stress. My aim is that these findings will contribute to understanding the importance of maternal care and the *postpartum* oxytocinergic activation that appears to exert beneficial effects through a mechanism involving the stress/anti-stress balance and the OT/BDNF interplay. Together, these findings suggest potential therapeutic strategies for mitigating the effects of early-life stress and improving the health of the mother-infant dyad.

## Academic background

- **PhD Candidate in Neuroscience** (oct.2021-sept.2024): Lille University; Funding University of Lille and Region Hauts de France
- **Laureate of a PhD research grant** in competition of the Doctoral School of Biology and Health in Lille (EDBSL) (July 2021)
- **Master degree in Biology-Health, specialization: Cellular, Integrative, and Translational Neurosciences** (2019-2021): Lille University
- **Bachelor in Cell Biology and Physiology** (2017-2019): Lille University
- Selected to study in France through the competitive **Campus France system**: July 2017
- **Bachelor in Microbiology** (2014-2017): University of Bejaia (Algeria)
- **Baccalaureate in Experimental Sciences** (2014): Mixed High school of Sidi Aich (Algeria)

## Internships

- **UGSF-UMR 8576 CNRS (France):** Master 2 Internship (6 months, 2021); GlycoStress team (Prof. S. Morley-Fletcher))/ Regulation of terminal glycosylation (Dr. A Harduin-Lepers)
- **UGSF-UMR 8576 CNRS (France):** Master 1 Internship (6 weeks, 2020); GlycoStress team (Prof. S. Morley-Fletcher)/ Regulation of terminal glycosylation (Dr. A Harduin-Lepers)
- **SCALab UMR 9193 (France):** Bachelor's Year 3 Internship (4 weeks, 2019); with Dr Cedrick Bonnet at the hospital Roger Salangro
- **Sidi Aich Hospital (Algeria):** Voluntary Internship in Microbiology and Biochemistry during my second Bachelor year (1 month 2016)
- **Tinebdar Polyclinic (Algeria):** Voluntary Internship in Microbiology and Biochemistry during my second Bachelor year (1 month 2016).

## Formations

- **Animal experimentation habilitation:** November 2021- February 2022, (EDBSL-Lille)
- **Citizen engagement in research (Ethics):** On May 6$^{th}$, (EDBSL-Lille)
- **The training for RNA-seq in Lille:** The 12$^{th}$ to the 17$^{th}$ of October 2022 (Bililleplatform, Lille)

# Scientific productions

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***Publications***

**R. Benlakehal**, A. Gaetano, H. Bouwalerh, V. Cogez, A. Harduin-Lepers, S. Maccari, S. Morley-Fletcher. *Limosilactobacillus reuteri* Supplementation in Lactating Rats Improves Maternal Behavior and Hypothalamic Oxytocin/ Brain-derived neurotrophic factor balance, Offering Insights for *Postpartum* Depression.*(Submitted to Biological Psychiatry Global Open Science)*.

S. Morley-Fletcher*A. Gaetano*, **R. Benlakehal**, M. Darnaudery, H. Bouwalerh, R Verhaeghe, G. Van Camp, I. Rocchi, C. Grare, JM. Lo Guidice, M. Szyf, S. Maccari. Transient *postpartum* activation of oxytocin receptor prevents postnatal intergenerational inheritance of early life stress. (*In preparation*).

**R. Benlakehal**, A. Gaetano, H. Bouwalerh, G. Van Camp, S. Maccari, S. Morley-Fletcher. Gestational stress in F0 dams induces a transgenerational inheritance in the stress/anti-stress balance of male F3 offspring, while peripheral and central oxytocinergic activation via carbetocin mitigate PRS deficits up to F3 *(In preparation)*.

**R. Benlakehal**, A. Gaetano, H. Bouwalerh, R. Charlet, B. Sendid, A. Harduin-Lepers, S. Maccari, S. Morley-Fletcher. Investigation of the transmission of the beneficial effect of *postpartum Limosilactobacillus reuteri* treatment in stressed dams on the PRS male offspring: OTR/BDNF and Sialylation interplay *(In preparation)*.

***Oral communications***

**R. Benlakehal**. *Study of maternal programming induced by maternal stress: Transgenerational transmission and oxytocin* - **Seminar talk 2023 at the UGSF**, 1st december 2023, Lille university.

**R. Benlakeha**l. *Study of maternal programming induced by maternal stress: Transgenerational transmission and oxytocin* - **André Verbert Day 2023 - EDBSL Doctoral Students' Conference,** 13th november 2023 - Lille University, France - ***(Winner of best presentation)***.

**R. Benlakehal**. *Study of maternal programming induced by maternal stress: Transgenerational transmission and oxytocin* - **EURON PhD days 2023** - 26th to 27 january, 2023. Maastricht University, Netherlands.

***Posters***

**R. Benlakehal,** A. Jacquemart, A. Gaetano, H. Bouwalerh, S. Maccari, A. Harduin-Lepers, S. Morley-Fletcher. *"Study of Maternal Programming Induced by Maternal Stress: Transgenerational Transmission and Oxytocin."* **6th SF-DOHaD 2023 Society Congress**, 16th to 17th November 2023, Rennes University, France.

**R. Benlakehal**, A. Jacquemart, A. Gaetano, H. Bouwalerh, S. Maccari, A. Harduin-Lepers, S. Morley-Fletcher. *"Study of Maternal Programming Induced by Maternal Stress: Transgenerational Transmission and Oxytocin."* **45th Colloquium of the Neuroendocrinology Society**, 27th to 29th September 2023, Rouen University, France. Travel grant by the society.

**R. Benlakehal**, A. Jacquemart, A. Gaetano, H. Bouwalerh, S. Maccari, A. Harduin-Lepers, S. Morley-Fletcher. *"Study of Maternal Mediated Mechanisms in the Epigenetic Programming Induced by Maternal Stress: Transgenerational Transmission and Oxytocin."* **NeuroFrance 2023**, 24th to 26th May, Lyon University, France.

**R. Benlakehal**, A. Harduin-Lepers, S. Morley-Fletcher. *"Study of Maternal Programming Induced by Maternal Stress: Transgenerational Transmission and Oxytocin."* **EURON PhD Days 2022**, 7th to 8th February 2022, Hasselt University, Belgium.

***Other contributions***

**Member of the local organizing committee of EURON PhD days 2024** in Lille University, France (June 2023- February 2024).

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**For my CSI jury (Comité de Suivi Individuel)**

I am thankful to Prof. Mohamed Kabbaj and Dr. Paolo Giacobini, members of the CSI jury, for their engagement with my work over the past three years and for guiding my experimental choices. Prof. Kabbaj encouraged me to delve deeper into the mechanisms underlying my studies, while Dr. Giacobini, with his extensive knowledge, initiated my investigation into the hypothalamus aspects. Your guidance and insights have been instrumental in shaping the trajectory this research took.

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*Finally, I dedicate this thesis to my late paternal grandfather, Moussa Benlakehal, who devoted his life to the pursuit of freedom. Your legacy and courage have been a constant source of inspiration throughout my research journey, deepening my understanding of your dedication and your impact on future generations.*

**For my experimental friends**

I wanted to conclude this acknowledgment by mentioning the rats *(Rattus norvegicus)* whose participation in this study was indispensable. Their contribution to scientific advancement is immeasurable, and we ensured their welfare was always a top priority, adhering to the highest ethical standards and respecting their role in our research.

"The greatest mistake in the treatment of diseases is that there are physicians for the body and physicians for the soul, although the two cannot be separated."

**Hans Selye's book "The Stress of Life" (1956)**

# List of important abreviations

**BDNF** - Brain-Derived Neurotrophic Factor

**CBT** - Carbetocin

**CORT** - Corticosterone

**DOHaD** - Developmental Origins of Health and Disease

**ELS** - Early-life stress

**EPM** - Elevated Plus Maze

**F0, F1, F2, F3** - Filial generations 0, 1, 2 and 3 (indicating different generations in the study)

**GCs** - Glucocorticoids

**GR** - Glucocorticoid Receptors

**HPA** - Hypothalamic-Pituitary-Adrenal

***L. reuteri* -** *Limosilactobacillus reuteri*

**mGluR2/3** - Metabotropic Glutamate Receptors 2 & 3

**MR** - Mineralocorticoid Receptors

**OT** - Oxytocin

**OTR** - Oxytocin Receptors

**PPD** - *postpartum* depression

**PRS** - Perinatal Stress

**PSA-NCAM** - Polysialylated Neural Cell Adhesion Molecule

**ST6GAL1** - Beta-galactoside alpha-2,6-sialyltransferase 1

**ST8SIA4** - Alpha-2,8-sialyltransferase 4

# Study of maternal-mediated mechanisms in the epigenetic programming induced by maternal stress:

# Transgenerational transmission and oxytocin

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# General Summary

Stress during critical developmental periods, such as the perinatal stages, significantly impacts health and contributes to disease development in offspring, rooted in Barker's DOHaD theory. Meaney and Szyf's seminal work highlighted how maternal behavior shapes patterns in offspring, impacting lifelong health outcomes. Maternal care variations, like those seen in the perinatal stress (PRS) model, influence HPA axis function and offspring stress reactivity. Epigenetic changes, such as GR gene hypermethylation in low maternal care rats, underscore the role of maternal behavior in stress programming.

Oxytocin (OT) plays a crucial role in promoting positive maternal behavior and reducing stress. Administration of OT or oxytocinergic agonists like carbetocin (CBT) in stressed lactating rats has shown to reverse long-term effects of PRS on gene expression and behavior, enhancing maternal care and correcting offspring developmental trajectories. Moreover, the gut-brain axis plays a significant role in stress response regulation. A probiotic like *Limosilactobacillus reuteri (L. reuteri)* can modulate the gut-brain axis, increasing oxytocin concentrations and improving social behavior. This intervention shows promise in mitigating the effects of stress-induced maternal dysfunction and normalizing HPA axis function.

In this context, this PhD thesis aims to study maternal-mediated mechanisms in the epigenetic programming induced by maternal stress, and deepen our understanding of the lasting impacts of early-life stress and its transmission across generations. It underscores the critical role of maternal behavior in this process and explores interventions like carbetocin and probiotics in reversing stress programming. By focusing on the *postpartum* period for intervention, this research contributes to developing targeted strategies to improve long-term health outcomes and break the cycle of stress vulnerability across generations. In this context, this thesis investigated in **chapter one** the long-term effects of PRS and the corrective effect of maternal OT, and in **chapter two** the maternal mechanisms of PRS transmission and its reversal by the probiotic *L. reuteri.*

**Chapter One: Epigenetic inheritance of the perinatal stress:** Investigations of the persistence of PRS-related deficits through the maternal line, in both intergenerational and transgenerational transmission manner, and highlight if the oxytocinergic activation through

pharmacologic approach in F0 dams during *postpartum* weeks was strong enough to correct PRS deficits across generations.

**A) Transient *postpartum* activation of oxytocin receptor prevents postnatal intergenerational inheritance of early life stress:** We have demonstrated that gestational stress in F0 dams reduces maternal behavior, which is transmitted intergenerationally to F1 PRS mothers. This stress also affects other parameters associated with maternal behavior, such as nest architecture and the maternal response to intruders, including CORT and OT balance in F0-F1 dams. These deficits manifest in the offspring, resulting in disrupted risk-taking behavior in the EPM and altered CORT/OT balance in both male and female PRS models, extending up to F2 offspring. Mechanistically, PRS males exhibit increased BDNF expression and reduced MR and GR gene expression, alongside imbalances in gene promoter methylation observed up to F2 males. To underscore the importance of maternal-mediated mechanisms, we administered *postpartum* CBT to correct maternal behavior in stressed F0 dams. Consistent with previous findings of the team we observed increased maternal behavior in CBT-treated dams. Remarkably, F0 CBT treatment effectively restored maternal behavior in F1 PRS dams, thereby correcting malprogramming in F1-F2 PRS offspring, specifically in terms of HPA axis reactivity, stress/anti-stress responses, and risk-taking behavior. Collectively, our study demonstrates the intergenerational transmission of stress in the PRS model and underscores the critical role of maternal behavior in mitigating and protecting against offspring malprogramming. Ultimately, we propose the *postpartum* period as a critical window for therapeutic interventions, advocating for OT as a promising approach to disrupt intergenerational inheritance and promote healthier developmental outcomes.

**B) Gestational stress in F0 dams induces a transgenerational inheritance in the stress/anti-stress balance of male F3 offspring, while peripheral and central oxytocinergic activation via carbetocin mitigate PRS deficits up to F3:** In this section, we highlighted the transgenerational transmission of PRS deficits from F0 to F3 generations, demonstrating that stress in F0 dams transmitted their malprogramming effects up to F2 mothers. The reduced maternal behavior observed in F2 dams impacted F3 male offspring, underscoring transgenerational transmission. Indeed, F3 PRS males exhibited reduced risk-taking behavior in the EPM and disruptions in CORT/OT balance. *Postpartum* treatments using intraperitoneal (IP) and intranasal (IN) CBT in F0 dams corrected maternal

behavior and increased maternal OT levels up to F2, resulting in reduced maternal CORT levels specifically in F0 dams but not in F2 dams. Furthermore, these F0 treatments rescued risk-taking behavior transgenerationally in F3 PRS males, along with their OT and CORT levels. This chapter clearly demonstrates a robust correlation between deficits in F0 dams and outcomes in F3 PRS males, highlighting the extensive impact of stress on behavior and neuroendocrinological parameters. Additionally, we propose OT as a therapeutic avenue for treating transgenerational transmission of early-life stress. This study is the first of its kind to mitigate transgenerational transmission and confirms the maternal capacity to program across multiple generations.

**Chapter Two**: **The anti-stress effect of maternal *Limosilactobacillus reuteri:*** aims to address the anti-stress effect of *Limosilactobacillus reuteri* in the perinatal stress model.

**A) *Limosilactobacillus reuteri* supplementation in lactating rats improves maternal behavior and hypothalamic Oxytocin/ Brain-Derived Neurotrophic Factor balance, offering insights for *postpartum* depression:** In this chapter, our focus was on the mothers and the oxytocinergic action of *L. reuteri* as an alternative (probiotic) strategy to revert the impact of gestational stress on maternal care and subsequently interfere with its transmission. Our findings reveal that *L. reuteri* effectively mitigated pronounced behavioral changes triggered by maternal (gestational) stress, including decreased maternal care and diminished reactivity to pups' separation, without affecting non-pup-oriented behaviors. Crucially, *L. reuteri* demonstrated modulatory effects on the OT/BDNF balance in the synaptosomes of the hypothalamus, evidenced by the reduction of elevated BDNF levels and an increase in OT concentrations in the plasma and hypothalamus of stressed dams, adjusting OTR forms in the hypothalamus of treated dams under stress. This study highlights the therapeutic potential of *L. reuteri* in alleviating the adverse effects of gestational stress on maternal behavior and physiology in the PRS rat model, offering insights into its molecular mechanisms and suggesting potential bio-pharmacological interventions to enhance maternal care in the context of *postpartum* depression (PPD).

**B) Investigation of the transmission of the beneficial effect of *postpartum Limosilactobacillus reuteri t*reatment in stressed dams on the PRS male offspring: OTR/BDNF and Sialylation interplay:** This chapter explores in the offspring the potential

reprogramming effect of maternal treatment with *Lactobacillus reuteri* and assesses whether the findings align with those observed using a direct agonist of oxytocin receptors, as demonstrated in the first chapter. We delve into parallel mechanisms beyond classical epigenetics, such as genes implicated in sialylation processes. After examining the corrective effects of *L. reuteri* on maternal behavior, we investigated whether this correction could effectively rescue behavioral, hormonal, and neurochemical deficits in male PRS offspring.

First, to assess the anti-stress effect of maternal probiotic treatment in the offspring, we focused on the stress hormone CORT and a key behavioral test, risk-taking behavior in the EPM. Second, considering the PRS model's established reduction in neuroplasticity and neurogenesis, we investigated BDNF as well as OTR protein expression. We also examined the expression of sialylation-related enzymes ST8SIA4 and ST6GAL1, which are crucial for brain plasticity through polysialylated neural cell adhesion molecules (PSA-NCAM) and inflammation. Indeed, the PRS model is characterized by hypo-neurogenesis and proinflammation. Additionally, the protein markers BDNF and OTR, which we selected for our study, are also targets of glycosylation.

In this last chapter we found that PRS reduced risk-taking behavior and increased CORT levels in male offspring; it also disturbed neurochemical parameters in the brain including BDNF, OTR, ST8SIA4 and ST6GAL1. The probiotic treatment in the mothers rescued risk-taking behavior in male PRS offspring, corrected CORT levels in the plasma but failed to mitigate the BDNF and OTR protein expression as well as gene expression of ST8SIA4 and ST6GAL1 in the ventral hippocampus.

Taken together, these PhD results indicate that **1)** intergenerational inheritance of early life stress is mediated by maternal oxytocin, **2)** PRS-related deficits get transmited transgenerationally to F3 males and *postpartum* carbetocin breaks the inheritance, **3)** *Postpartum L. reuteri* corrects maternal behavior through a mechanism that involves BDNF and OT balance in the hypothalamus, **4)** PRS programing in the offspring partially-mitigated by *postpartum L. reuteri* treatment in the mothers.

*Overall, this PhD thesis aims to strengthen the understanding of early life stress lasting blueprint, and its transmission across multiple generations, with maternal behavior as a key mediator in this programming and transmission. Importantly, this work contributes to reveal*

*promising therapeutic avenues targeting the postpartum period using probiotic or carbetocin both targeting the oxytocinergic system, showing the potential for reversing stress programming, underscoring the epigenetic aspect of early life stress.*

# Résumé général

Le stress pendant les périodes critiques du développement, telles que les stades périnataux, impacte significativement la santé et contribue au développement des maladies chez la progéniture, enraciné dans la théorie DOHaD de Barker. Le travail séminal de Meaney et Szyf a mis en lumière comment le comportement maternel façonne les schémas chez la progéniture, impactant les résultats de santé à vie. Les variations des soins maternels, comme celles observées dans le modèle de stress périnatal (PRS), influencent la fonction de l'axe HPA et la réactivité au stress de la progéniture. Les changements épigénétiques, tels que l'hyperméthylation du gène GR due à un faible soin maternel, soulignent le rôle du comportement maternel dans la programmation du stress.

L'ocytocine (OT) joue un rôle crucial en favorisant un comportement maternel positif et en réduisant le stress. L'administration d'ocytocine ou d'agonistes ocytocinergiques comme le carbetocin chez des rates allaitantes stressées a montré qu'elle inversait les effets à long terme du PRS sur l'expression génique et le comportement, améliorant les soins maternels et corrigeant les trajectoires développementales de la progéniture. De plus, l'axe intestin-cerveau joue un rôle significatif dans la régulation de la réponse au stress. Les probiotiques comme *Limosilactobacillus reuteri* peuvent moduler l'axe intestin-cerveau, augmentant les niveaux d'ocytocine et améliorant le comportement social. Cette intervention montre des promesses pour atténuer les effets de la dysfonction maternelle induite par le stress et normaliser la fonction de l'axe HPA.

Dans ce contexte, cette thèse vise à étudier les mécanismes médiés par la mère dans la programmation épigénétique induite par le stress maternel, et approfondir notre compréhension des impacts durables du stress précoce et de sa transmission à travers les générations. Elle souligne le rôle critique du comportement maternel dans ce processus et explore des interventions comme le carbetocin et les probiotiques pour inverser la programmation du stress. En se concentrant sur la période *postpartum* pour l'intervention, cette recherche contribue au développement de stratégies ciblées pour améliorer les résultats de santé à long terme et briser le cycle de la vulnérabilité au stress à travers les générations. En résumé, cette étude démontre dans deux chapitres principaux les résultats suivants.

**Chapitre 1**: Héritage épigénétique du stress périnatal : Investigation de la persistance des déficits liés au PRS à travers la lignée maternelle, tant dans la transmission intergénérationnelle que transgénérationnelle, et mise en lumière de la puissance de l'activation ocytocinergique par une approche pharmacologique chez les femelles F0 pendant les semaines *postpartum* pour corriger les déficits du PRS à travers les générations.

**A) Activation *postpartum* transitoire des récepteurs d'ocytocine prévient l'héritage intergénérationnel du stress précoce** : Nous avons démontré que le stress gestationnel chez les femelles F0 réduit le comportement maternel, transmis intergénérationnellement aux mères F1 PRS. Ce stress affecte également d'autres paramètres associés au comportement maternel, tels que l'architecture du nid et la réponse maternelle aux intrus, y compris l'équilibre CORT et OT chez les femelles F0-F1. Ces déficits se manifestent chez la progéniture, entraînant un comportement de prise de risque perturbé dans le labyrinthe en plus (EPM) et un déséquilibre CORT/OT chez les modèles PRS mâles et femelles, s'étendant jusqu'à la progéniture F2. Mécaniquement, les mâles PRS montrent une expression accrue de BDNF et une réduction de l'expression des gènes MR et GR, ainsi que des déséquilibres dans la méthylation du promoteur génique observée jusqu'aux mâles F2. Pour souligner l'importance des mécanismes médiés par la mère, nous avons administré du CBT *postpartum* pour corriger le comportement maternel chez les femelles F0 stressées. Conformément aux résultats de Gatta et al., nous avons observé un comportement maternel accru chez les femelles traitées au CBT. Remarquablement, le traitement CBT chez les femelles F0 a efficacement restauré le comportement maternel chez les femelles F1 PRS, corrigeant ainsi la mal-programmation chez la progéniture F1-F2 PRS, notamment en termes de réactivité de l'axe HPA, de réponses au stress/anti-stress et de comportement de prise de risque. Dans l'ensemble, notre étude démontre la transmission intergénérationnelle du stress dans le modèle PRS et souligne le rôle critique du comportement maternel pour atténuer et protéger contre la mal-programmation de la progéniture. En définitive, nous proposons la période *postpartum* comme une fenêtre critique pour les interventions thérapeutiques, en préconisant l'ocytocine comme approche prometteuse pour perturber l'héritage intergénérationnel et promouvoir des résultats développementaux plus sains.

**B) Le stress gestationnel chez les femelles F0 induit un héritage transgénérationnel dans l'équilibre stress/anti-stress des mâles F3, tandis que l'activation ocytocinergique**

**périphérique et centrale via le carbetocin atténue les déficits PRS jusqu'aux F3** : Dans cette section, nous avons mis en lumière la transmission transgénérationnelle des déficits PRS des générations F0 à F3, démontrant que le stress chez les femelles F0 a transmis leurs effets de mal-programmation jusqu'aux mères F2. Le comportement maternel réduit observé chez les mères F2 a impacté la progéniture mâle F3, soulignant la transmission transgénérationnelle. En effet, les mâles PRS F3 ont montré un comportement de prise de risque réduit dans le labyrinthe en plus (EPM) et des perturbations dans l'équilibre CORT/OT. Les traitements *postpartum* utilisant le CBT par voie intrapéritonéale (IP) et intranasale (IN) chez les femelles F0 ont corrigé le comportement maternel et augmenté les niveaux d'OT maternel jusqu'aux F2, entraînant une réduction des niveaux de CORT maternel spécifiquement chez les femelles F0 mais pas chez les femelles F2. De plus, ces traitements F0 ont rétabli le comportement de prise de risque de manière transgénérationnelle chez les mâles PRS F3, ainsi que leurs niveaux d'OT et de CORT. Ce chapitre démontre clairement une corrélation robuste entre les déficits chez les femelles F0 et les résultats chez les mâles PRS F3, mettant en évidence l'impact étendu du stress sur le comportement et les paramètres neuro-endocrinologiques. De plus, nous proposons l'OT comme une voie thérapeutique pour traiter la transmission transgénérationnelle du stress précoce. Cette étude est la première du genre à atténuer la transmission transgénérationnelle et confirme la capacité maternelle à programmer à travers plusieurs générations.

**Chapitre 2**: L'effet anti-stress de *Limosilactobacillus reuteri* : vise à aborder l'effet anti-stress de *Limosilactobacillus reuteri* dans le modèle de stress périnatal.

**A) Supplémentation en *Limosilactobacillus reuteri* chez les rates allaitantes améliore le comportement maternel et l'équilibre hypothalamique entre l'ocytocine et le facteur neurotrophique dérivé du cerveau, offrant des perspectives pour la dépression *postpartum***: Dans ce chapitre, notre focus était sur les mères et l'action ocytocinergique de *L. reuteri* sur le comportement maternel en tant que stratégie alternative (probiotique) pour inverser l'impact du stress maternel sur les soins maternels et interférer ensuite avec sa transmission. Nos résultats révèlent que *L. reuteri* atténuait efficacement les changements comportementaux prononcés déclenchés par le stress maternel, y compris la diminution des soins maternels et la réduction de la réactivité à la séparation des petits, sans affecter les comportements non orientés vers les petits. Crucialement, *L. reuteri* a démontré des effets

modulateurs sur l'équilibre OT/BDNF dans les synaptosomes de l'hypothalamus, comme en témoigne la réduction des niveaux élevés de BDNF et l'augmentation des niveaux d'OT chez les femelles stressées, ajustant les formes de OTR chez les femelles sous stress traitées. Cette étude met en lumière le potentiel thérapeutique de *L. reuteri* pour atténuer les effets adverses du stress gestationnel sur le comportement maternel et la physiologie dans un modèle de rat PRS, offrant des perspectives sur ses mécanismes moléculaires et suggérant des interventions bio-pharmacologiques potentielles pour améliorer les soins maternels dans le contexte de la dépression *postpartum* (DPP).

**B) Investigation de la transmission de l'effet bénéfique du traitement *postpartum* à *Limosilactobacillus reuteri* chez les femelles stressées sur la progéniture mâle PRS : Interaction OTR/BDNF et sialylation :** Ce chapitre explore dans la progéniture l'effet de reprogrammation potentiel du traitement maternel avec *Lactobacillus reuteri* et évalue si les résultats correspondent à ceux observés en utilisant un agoniste direct des récepteurs d'ocytocine, comme démontré par des travaux antérieurs de l'équipe. Nous nous sommes penchés sur des mécanismes parallèles au-delà de l'épigénétique classique, tels que les gènes impliqués dans les processus de sialylations. Après avoir examiné les effets correctifs de *L. reuteri* sur le comportement maternel, nous avons étudié si cette correction pouvait efficacement rescaper les déficits comportementaux, hormonaux et neurochimiques chez la progéniture mâle PRS. Premièrement, pour évaluer l'effet anti-stress du traitement probiotique maternel chez la progéniture, nous nous sommes concentrés sur l'hormone de stress CORT et sur un comportement clé de réponse au stress, la prise de risque dans le labyrinthe en croix surélevée (EPM). Deuxièmement, considérant la réduction établie dans le modèle PRS de la neuroplasticité et de la neurogenèse, comme en témoigne la diminution de PSA-NCAM et des niveaux perturbés de BDNF, nous avons étudié l'expression des protéines BDNF et OTR. Nous avons également examiné l'expression des enzymes liées à la sialylation ST8SIA4 et ST6GAL1, cruciales pour la plasticité cérébrale à travers les molécules d'adhésion cellulaire neuronale polysialylées (PSA-NCAM) et impliquées dans l'inflammation. En effet, le modèle PRS se caractérise par une hypo-neurogenèse et une pro-inflammation. De plus, les marqueurs protéiques BDNF et OTR, sélectionnés pour notre étude, sont également des cibles de glycosylation.

Dans ce dernier chapitre, nous avons constaté que le PRS réduisait le comportement de prise de risque et augmentait les niveaux de CORT chez la progéniture mâle ; il perturbait également les paramètres neurochimiques dans le cerveau, y compris le BDNF, l'OTR, ST8SIA4 et ST6GAL1. Le traitement probiotique chez les mères a récupéré le comportement de prise de risque chez la progéniture mâle PRS, corrigé les niveaux de CORT dans le plasma mais n'a pas atténué l'expression des protéines BDNF et OTR ainsi que l'expression des gènes des enzymes de sialylation ST8SIA4 et ST6GAL1 dans l'hippocampe ventral.

Pris dans leur ensemble, ces résultats de thèse indiquent que **1)** l'héritage intergénérationnel du stress précoce est médié par l'ocytocine maternelle, **2)** les déficits liés au PRS sont transmis de manière transgénérationnelle aux descendants F3 et la carbétocine *postpartum* rompt la transmission, **3)** *L. reuteri postpartum* corrige le comportement maternel par un mécanisme impliquant l'équilibre BDNF-OT dans l'hypothalamus, **4)** la programmation du PRS dans la progéniture est partiellement atténuée par le traitement *postpartum* à la *L. reuter*i chez les mères.

En conclusion, cette thèse vise à renforcer la compréhension du schéma durable du stress précoce et de sa transmission à travers plusieurs générations, avec le comportement maternel comme médiateur clé de cette programmation et transmission. De manière importante, ce travail contribue à révéler des avenues thérapeutiques prometteuses ciblant la période *postpartum* à l'aide de probiotiques ou de carbetocin, montrant le potentiel de renverser la programmation du stress, soulignant l'aspect épigénétique du stress précoce.

# General Introduction

# General Introduction

## I. Early life programming and the DOHaD theory

For decades, scientists believed that genetics alone determined an individual's health. However, major breakthroughs have revealed a complex interaction between genes and the environment, leading to the debate about the influence of genetics (nature) versus environment (nurture) on human development. Francis Galton, influenced by his cousin Charles Darwin, famously examined this concept in the late 19th century, focusing on the relative impact of heredity and environment (Galton, 1914 republication of Galton, 1869). In 1942, British developmental biologist Conrad Waddington coined the term "epigenetics" which refers to phenotypic changes not caused by DNA sequence alterations but transmissible to offspring (Waddington, 1942). Professor of cell and developmental biology Shelley Berger later defined epigenetics as a *" stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"* (Berger et al., 2009). Epigenetic mechanisms typically involve DNA methylation, histone modifications, and noncoding RNAs, including microRNAs (Tsankova et al., 2007).

To elucidate the influence of environmental factors, pioneering researchers investigated these mechanisms, providing substantial insights into the biological processes underlying early life influence. Furthermore, epidemiological studies corroborated Waddington previous work, in the aftermath of World War I, the epidemiologist David Barker and his collaborators proposed that poor prenatal conditions increase the risk of cardiovascular disease in adults. They identified a strong correlation between ischemic heart disease mortality rates and the geographical location in England and Wales, noting higher prevalence in northern industrial towns and poorer rural areas compared to the south and east (Barker and Osmond, 1986), this early work laid the foundation for understanding *health programming*, where environmental factors shape health outcomes through biological mechanisms. Subsequently, Barker and his team highlighted that gestational undernutrition was associated with increased risks of neonatal death, and the few survivors of poor prenatal conditions presented elevated risks of developing ischemic heart disease later in life (Barker, 1995a). Accordingly, adverse early life environments, such as poor nutrition during critical developmental periods, increase

susceptibility to chronic diseases like ischemic heart disease, demonstrating that individuals with the lowest birth weights had the highest mortality ratios from ischemic heart disease (Barker, 1995b).

Barker's work later evolved into the **theory of Developmental Origins of Health and Disease (DOHaD),** which states that environmental factors during prenatal and early postnatal development can significantly impact long-term health (Barker, 2004). However, if adverse exposure continues after the birth of the progeny, there is a low possibility of maladaptive programming. This is because both the in-utero and postnatal environments remain unchanged, as demonstrated by the Russian cohort studies, referred also as the Siege of Leningrad (now Saint Petersburg). This cohort represents one of the most extreme examples of prolonged starvation and hardship experienced by the Russian population during World War II (Stanner et al., 1997). Beyond cardiovascular disease programming, animal models with early adverse experiences are prone to a higher risk of developing stress-related disorders in adulthood that share great similarity to stress-related disease in humans such as metabolic disorders, addiction, and circadian rhythms alterations thus, underscoring the long-term effects of prenatal and early-life environments on mental health (Maccari et al., 2003).

*Many contemporary studies trace back to Barker's pioneering work including neuroscience. In 1995, Barker noted, "We are beginning to understand something of the mechanisms underlying these associations. The programming of blood pressure, insulin responses to glucose, cholesterol metabolism, blood coagulation, and hormonal settings are all areas of active research" (Barker, 1995a), This foresight remains relevant today, as ongoing research strives to elucidate these mechanisms. Several scientific fields based their work on the DOHaD theory, and more specifically in the field of stress and early-life stress (ELS).*

## II. Historical perspectives on stress and the emergence of early-life stress (ELS) research

Over the past decades, the DOHaD theory, which initially focused on cardiovascular disease research, has expanded to explore the broader factors of early life experiences, including stress. This foundational understanding laid the groundwork for exploring how early life

stress (ELS) can program lifelong changes in stress reactivity and contribute to susceptibility to a range of physical and mental health disorders (McEwen, 2012).

Stress, defined by the World Health Organization as a state of mental tension or worry triggered by challenging circumstances, has evolved from its historical roots to a physiological concept. In 1915, Walter Cannon introduced the concept of "stress" with the fight-or-flight response, describing the body's rapid physiological reaction to perceived threats, preparing it to either confront or escape danger by releasing stress hormones like adrenaline. This primal response boosts heart rate, blood pressure, and glucose levels, providing the energy needed for immediate action (Cannon, 1915). Hans Selye, an endocrinologist often referred to as the "father of stress research," proposed the General Adaptation Syndrome (GAS), describing the body's response to stress in three stages: alarm, resistance, and exhaustion (Selye, 1936). Selye's theory stemmed from his observations of patients with various chronic illnesses who exhibited common symptoms such as fatigue, loss of appetite, and weight loss. These findings led him to conclude that these symptoms represented a generalized response to stress rather than being specific to any particular disease (Selye, 1936). Following this, Selye conducted pioneering experiments involving placing rats in stressful situations and observing consistent physiological responses such as adrenal hyperactivity, lymphatic atrophy, and peptic ulcers. These experiments were among the first to identify the hypothalamic-pituitary-adrenal (HPA) axis (Selye, 1952). During his investigation, he collected various extracts from cow ovaries and he injected them into female rats, and measured their responses. His autopsies revealed three surprising findings: enlargement of the adrenal glands, atrophy of the lymphatic system including the thymus, and peptic ulcers in the stomach and duodenum. These effects were consistent across all noxious agents injected. Further experiments involved placing the rats in stressful situations, like on a cold roof or a revolving treadmill. The results remained the same: adrenal hyperactivity, lymphatic atrophy, and peptic ulcers (Selye, 1936; Tan and Yip, 2018). Selye broke down the term stress into "distress" (negative stress) and "eustress" (positive stress) to differentiate between stress responses triggered by negative, unpleasant stressors and those caused by positive, stimulating factors. He recognized that stress is not always harmful and can have beneficial effects depending on its nature and duration. Selye was known for his innovative approach to naming and conceptualizing ideas, although his usage of these terms was not

always consistent (Selye, 1976). Since 1915, both Cannon and Selye's research significantly advanced our understanding of stress physiology and its effects on health (Rochette et al., 2023; Tan and Yip, 2018).

*However, stress has affected humans for millennia, as demonstrated by cortisol levels in archeological hair of pre-Columbian Peru, suggesting its impact for at least 1500 years (Webb et al., 2010). Understanding the mechanisms underlying the impact of ELS is crucial for elucidating how early life experiences shape later health outcomes. To gain deeper insights, it is essential to conduct more comprehensive studies on human cohorts exposed to early life stress, identifying specific biological, psychological, and social factors that mediate these effects. This holistic approach will inform the development of interventions aimed at promoting resilience and improving long-term health outcomes for individuals affected by early life stressors.*

## 1. Cohort studies on Early-Life Stress (ELS): Insights from historical adversities

Over the years, scientists have used cohorts to investigate Early-Life Stress (ELS), drawing on historical events and epidemiological contributions. These cohorts have proven invaluable in advancing our understanding and solidifying the developmental programming.

One of the most studied cohorts in the context of the DOHaD theory is the Dutch Hunger Winter cohort which covers individuals born during late 1944 to early 1945. During the famine, the caloric intake was around 600 calories, but not similar for all individuals; rather, it varied based on availability and personal circumstances. Here, individuals exposed to the famine while in utero exhibited decreased glucose tolerance later in life, affecting both men and women (Lumey et al., 2011; Ravelli et al., 1998). One of the most interesting and unique findings in this cohort is that it shows perfectly the importance of late pregnancy exposure when no differences were found in individuals exposed during the first semester of pregnancy. Indeed, late pregnancy exposure induced problems in terms of birth size, body proportions (Stein et al., 2004), and glucose intolerance (Ravelli et al., 1998). Also, maternal malnutrition had an influence on cognitive function in later life as suggested by a lower performance of men and women *in utero* during the famine (Roseboom et al., 2011). Furthermore, individuals prenatally exposed to famine during the Dutch Hunger Winter

exhibited, six decades later, reduced DNA methylation of the IGF2 gene compared to their unexposed same-sex siblings (Heijmans et al., 2008).

Similarly, the Russian Famine cohort, particularly during the Siege of Leningrad from 1941 to 1944, offers another perspective on the effects of prolonged starvation. Indeed, three to six decades after the siege, men showed increased blood pressure and mortality from ischemic heart disease and stroke, highlighting the enduring health impacts of severe nutritional deprivation during critical developmental periods (Sparén et al., 2004).

Building on previous foundational research, scientists have also conducted significant studies on Holocaust survivors, which serve as a prominent case study of posttraumatic stress disorder (PTSD); these investigations revealed a higher prevalence of lifetime PTSD, mood and anxiety disorders, as well as substance abuse disorders, in the offspring of Holocaust survivors compared to control groups (Yehuda et al., 2008). Another study on aged Holocaust survivors showed that PTSD symptom severity generally diminishes over time, however in some cases, there was a delayed onset or tended to show a worsening in PTSD (Yehuda et al., 2009). Furthermore, at the molecular level, cortisol levels in Holocaust survivors fluctuated over a 10-year period, increasing in those with remitted PTSD and declining in those who developed or maintained PTSD. Cortisol levels at baseline were predictive of changes in diagnostic status, underscoring their significance in PTSD progression (Yehuda et al., 2007). At the epigenetic level, Holocaust exposure altered FKBP5 methylation in survivors and their offspring, precisely survivors showing higher methylation levels at bin 3/site 6 compared to controls, while their offspring exhibited lower levels (Yehuda et al., 2016). In this context, FKBP5 is involved in regulating the stress response system, particularly the glucocorticoid receptor (GR) signaling pathway. It plays a role in modulating the sensitivity of cells to stress hormones like cortisol, indeed offspring with paternal PTSD due to Holocaust showed higher GR-1F promoter methylation (Yehuda et al., 2014). In addition, combat veterans with PTSD showed a higher DNA methylation of four CpG sites at the BDNF promoter compared with those without PTSD, as well as alcohol problems (Kim et al., 2017). The previous clinical findings were concordant in animal models, indeed, early maltreatment in rats produced persisting changes in DNA methylation of BDNF that also persisted in the offspring of maltreated rats (Roth et al., 2009). Additionally, further investigation showed that environmental factors impact other epigenetic mechanisms including histone modifications

and miRNA expression in both human and animal models (Bale et al., 2010; Skvortsova et al., 2018).

A more recent cohort, the Quebec Ice Storm of January 1998, offers a compelling case study of acute environmental stressors and their impact on child development. This cohort includes families affected by the massive ice storm that struck Quebec (Canada), from January 4-10, 1998. The storm caused widespread power outages, extreme cold, and significant infrastructure damage. Studies conducted on this cohort, collectively known as Project Ice Storm (King and Laplante, 2005), shed light on the consequences of this natural disaster, which left thousands of Canadians without power and access to essential services. Building upon the project ice storm, research has shown that in the children of mothers who experienced this natural disaster, exhibited disturbed levels of insulin and C-peptide secretion (Dancause et al., 2013; Fernandez-Twinn et al., 2019; King and Laplante, 2005). Moreover, a 2023 study emphasized a correlation between the broad autism phenotype in young adults and women who were pregnant on January 9, 1998, or who conceived within the subsequent three months (Li et al., 2023). The Project Ice Storm studies further illustrated the profound impact of prenatal maternal stress on offspring through epigenetic changes. For instance, 13 years after the Quebec ice storm, children of mothers exposed to the disaster exhibited DNA methylation changes in immune-related genes (Cao-Lei et al., 2015). Additionally, a 2015 study found DNA methylation deficits observed in this cohort affected BMI (Body Mass Index) and central adiposity in children (Cao-Lei et al., 2015), as well as patterns related to diabetes in children (Cao-Lei et al., 2018).

*Collectively, findings from previous cohort studies underscore the profound and enduring effects of ELS and environmental exposures on health outcomes across lifespan. By elucidating the mechanisms underlying these effects, researchers can develop targeted interventions aimed at mitigating the negative consequences of ELS, promoting resilience in vulnerable populations, and understanding epigenetic mechanisms in the context of stress and developmental programming. This highlights epigenetics' significant contribution to the DOHaD framework, providing a biological basis for how early environmental factors influence long-term health outcomes.*

## 2. How epigenetics enhanced the credibility of the DOHaD concept

The exploration of epigenetics has fundamentally bolstered the DOHaD concept by emphasizing the critical role of early environmental factors in shaping neurogenic processes during adulthood. This paradigm shift underscores the profound implications for promoting cognitive health and resilience across the lifespan. Meaney and Szyf introduced ***the seductive allure of behavioral epigenetics to the field of neuroscience*** (Miller, 2010), profoundly shaping our understanding of how early environmental influences, particularly maternal care, imprint lasting changes on gene expression and neurodevelopment. Their seminal contributions, including their highly cited 2004 article in the journal *Nature Neuroscience*, have left an indelible mark on the field. In this work, they proposed the concept that variations in maternal behavior could alter DNA methylation patterns in offspring, thereby influencing brain function and behavior across generations (Weaver et al., 2004). This pioneering idea revolutionized neurosciences by demonstrating the dynamic interplay between environmental factors and epigenetic mechanisms in shaping lifelong health outcomes (Meaney, 2001; Miller, 2010; Szyf, 2022; Weaver et al., 2004).

Research in humans has demonstrated that elevated maternal psychological distress during pregnancy is linked to alterations in fetal brain structure and function, which persist in the offspring for months or even years post-birth. Moreover, such maternal distress is associated with adverse child neurodevelopmental outcomes and neuropsychiatric dysfunction (Wu et al., 2024). Studies, on a more recent event such as the COVID-19 pandemic, have shown that pregnant women infected with SARS-CoV-2 can lead to brain damage and post-birth psychiatric disorders in offspring (Wang et al., 2022). At the molecular levels of the brain, participants who went through PTSD due to trauma showed modified methylation profiles in the blood and prefrontal cortex (Logue et al., 2020), this phenomenon was also observed in studies on childhood maternal care, which found an association with DNA methylation of genes for brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OTR) in the peripheral blood cells of adult men and women (Unternaehrer et al., 2015). BDNF promoter I methylation showed positive correlations between post-mortem human peripheral and brain tissues (Stenz et al., 2015), opening new hope for easy translational studies.

Nevertheless, it is easier to highlight these brain epigenetic modifications in preclinical models. Indeed a prenatal stress model in mice showed brain programming effects accompanied by epigenetic modifications, offspring born from stressed mothers (PRS mice) had increased levels of DNMTs (DNA Methyltransferase 1 and 3a) in the GABAergic neuron of the hippocampus and frontal cortex, importantly, the overexpression of DNMTs was associated with a decrease in reelin (a large extracellular matrix glycoprotein) and Glutamate decarboxylase (GAD67) genes, those genes are responsible for brain development and function, and their dysregulation can contribute to the pathophysiology of various mental health disorders (Matrisciano et al., 2013). Indeed, these epigenetic changes were similar to changes observed in the post-mortem brains of psychiatric patients with GAD67 and reeling significantly decreased in patients with schizophrenia or bipolar disorder (Guidotti et al., 2000; Matrisciano et al., 2013). Moreover, the ultimate proof of brain programming is reflected in behavioral changes, as behavior is *fundamentally* controlled by the brain. Mice subjected to prenatal stress exhibited hyperactivity and significant deficits in social interaction, prepulse inhibition, and fear conditioning (Matrisciano et al., 2013). On the other hand, DNA methylation changes in the maternal oxytocin (OT) gene locus during pregnancy predict *postpartum* maternal intrusiveness (Toepfer et al., 2019). A study using Brandt's voles showed that environmental stress such as high housing density induced more aggressive behavior and increased expression of mRNA and protein of AVP and its receptor (AVPR), but decreased expression of mRNA and protein of OT and OT receptor in specific brain regions of voles (HUANG et al., 2021). In rodents, early maltreatment produced persisting changes in methylation of BDNF DNA that caused altered BDNF gene expression in the adult prefrontal cortex (Roth et al., 2009). Conversely, Weaver and colleagues demonstrated that increased pup licking and grooming (LG) and arched-back nursing (ABN) by rat mothers led to alterations in the offspring's epigenome at a glucocorticoid receptor (GR) gene promoter in the hippocampus (Weaver et al., 2004). Finally, a 2023 review by Dee et al, summarized that chronic stress exposure induces epigenetic changes in genes involved in neuronal development and hormonal regulation, such as GR and BDNF in rats, and OTR and SLC6A4 in humans. These epigenetic modifications are associated with increased susceptibility to mental health disorders, including depression, anxiety, personality disorders, and PTSD (Dee et al., 2023).

*Taken together, this last study and the previous ones mentioned above highlight the direct impact of environmental stress on the brain, within major brain regions such as the cortex, the hippocampus, through epigenetic mechanisms. Despite the reversible nature of epigenetic changes, the persistence of stress outcomes into adulthood and after highlights the potential for exploring how early life stressors may be transmitted across generations.*

## 3. How far can early-life programming get transmitted?

Epigenetic inheritance can occur in two distinct ways. The first is intergenerational transmission, which refers to the transfer of effects from one generation to the next, when both generations are directly exposed to an environmental factor. In contrast, transgenerational transmission involves the transfer of effects across multiple generations, where subsequent generations exhibit impacts despite not being directly exposed to the original environmental factor (Breton et al., 2021; Klengel et al., 2016). In gestating females (representing the F0 generation), environmental stressors can significantly impact both the fetus (F1) and the developing germ cells within the fetus itself (F2), leading to deficits transmitted through the ***maternal line***, this process is classified as intergenerational transmission, referring to the effects observed in the immediate exposed generations (from F0 to F2) (Breton et al., 2021; Klengel et al., 2016). Surprisingly, the effects of environmental stressors can also get passed down through generations without direct exposure even in the absence of the stressors, impacting the F3 and subsequent generations, this type of transmission is defined as transgenerational transmission, (Dee et al., 2023). Overall, the complexity of this phenomenon is briefly represented in **Figure 1**.

Moreover, if environmental stress occurs before gestation, the F0 generation (the directly exposed individual) and the F1 generation (germ cells within the F0) are impacted intergenerationally, when the transgenerational effects appear in the F2 generation, as this generation has not been directly exposed but still exhibits the impacts of the initial stressor. This means that the period of stress exposure determines whether the effects are intergenerational or transgenerational. According to Skinner (2008), epigenetic transgenerational phenotypes are those that persist in subsequent generations even when the initial environmental stressor is no longer present (Skinner, 2008). Nevertheless, not only the period and time of stress exposure can define the type of transmission, but also the parental

line exposed to the stressor, indeed the previous example focused on the maternal line, but studies exist also on the ***paternal line*** where the apparition of transgenerational transmission occures earlier (from F2), because males do not carry the fetus, which means they do not also carry the fetus' germ cells. However, there is still a notable misuse and confusion in the scientific field between the terms intergenerational and transgenerational transmission.

Often, studies that observe changes in the F2 generation mistakenly label these effects as transgenerational, when in fact they are intergenerational since the germ cells of the F1 generation were directly exposed to the initial stressor (Skinner, 2008). This distinction is crucial in understanding how environmental factors can propagate through generations and the mechanisms behind these transmission effects. Research has provided compelling evidence for these mechanisms, highlighting the role of epigenetic modifications such as DNA methylation, histone modification, and microRNAs (miRNA) in these processes. Pioneering studies have investigated these mechanisms to elucidate how environmental exposures can lead to long-term changes in gene expression and phenotype (Cao-Lei et al., 2015; Gapp et al., 2014; Unternaehrer et al., 2015; Zhang et al., 2013).

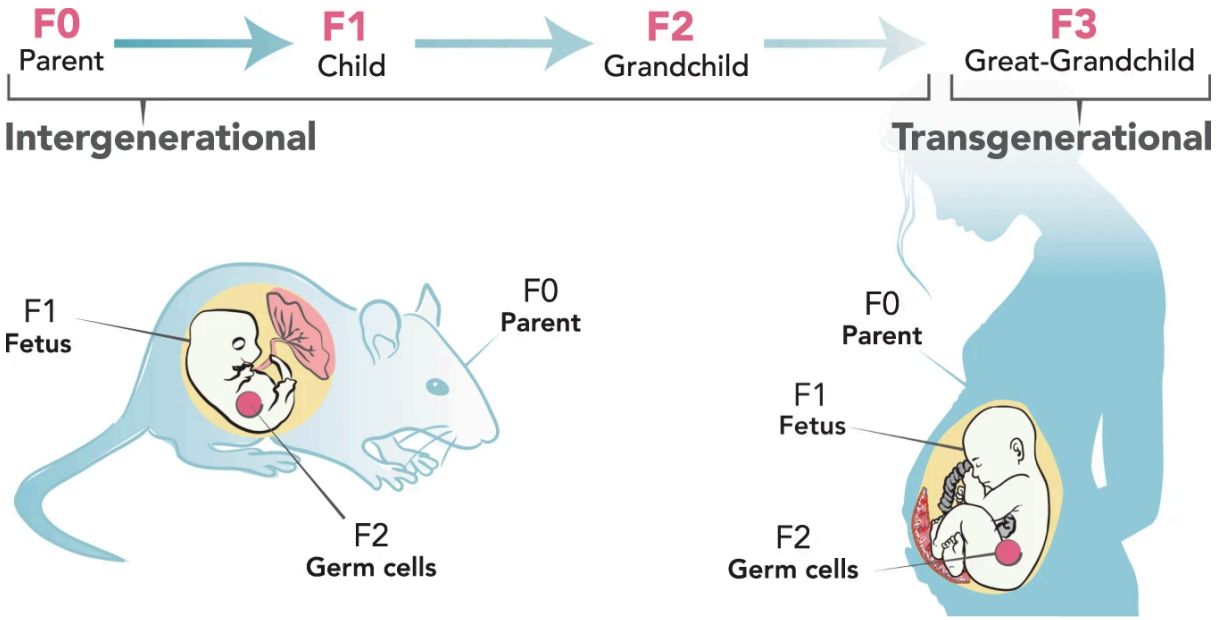


**Figure 1. Schematic explanation on the difference between intergenerational and transgenerational transmission when the studied parent is the female (maternal line) figure adapted from (Breton et al., 2021).**

## 4. Evidence of intergenerational and transgenerational transmission in the literature

Intergenerational transmission is one of the classical inheritances and the most studied one in the literature, as an example the programming that occurs in the offspring due to adverse environment perceived by the mother is intergenerational inheritance. Most human evidence of transmissions are intergenerational; indeed research consistently shows that offspring of highly stressed or traumatized parents are at increased risk for mental and physical health challenges. The concept of intergenerational trauma was introduced in the psychiatric literature through descriptions of behavioral and clinical problems in offspring of Holocaust survivors (Rakoff, 1966; Weinfeld et al., 1981). In a pivotal paper describing three patients who presented for psychiatric treatment, Rakoff wrote: "The parents are not broken conspicuously, yet their children, all of whom were born after the Holocaust, display severe psychiatric symptomatology. It would almost be easier to believe that they, rather than their parents, had suffered the corrupting, searing hell" (Rakoff, 1966). More recently, adult children of Holocaust survivors are more prone to PTSD, depression, and anxiety disorders, despite being born after World War II (Yehuda et al., 2008). Similarly, maternal PTSD resulting from trauma during pregnancy, such as the events of 9/11, correlates with adverse effects in offspring, with a notable trimester-specific impact suggesting *in utero* biological influences (Yehuda et al., 2005). In terms of physical health outcomes, maternal stress and PTSD during pregnancy, including exposure to psychosocial stress and trauma, have been linked to impaired uterine blood flow, low birth weight, and preterm birth (Berkowitz et al., 2003; Christiaens et al., 2015; Yonkers et al., 2014). These factors are linked to an increased risk of hypertension, insulin resistance, type 2 diabetes, and cardiovascular disease in adult offspring (Barker, 1995b; Ravelli et al., 1998). Moreover, parental Holocaust exposure has been linked with similar health issues in offspring, including hypertension, dyslipidemia, Type 2 diabetes, and overall poorer health (Bowers and Yehuda, 2016; Flory et al., 2011).

Recent studies on animals have provided insights into the intergenerational effects of parental experiences on offspring behavior and physiology. In a mouse model of postnatal maternal separation combined with unexpected stress, behavioral deficits such as impaired exploratory and risk-taking behavior, additionally to reduced weight in the offspring which was transmitted to the following generation through females (Weiss et al., 2011), similarly this

model presented stable intergenerational transmission of epigenetic deficits through the males (Franklin et al., 2010; Gapp et al., 2014). Furthermore, parental olfactory experiences can influence behavioral responses across generations through conditioned fear responses to specific odors , suggesting potential interventions to mitigate inherited behavioral changes (Debiec and Sullivan, 2014; Dias and Ressler, 2014). These studies collectively highlight the complexity of intergenerational transmission in animal models.

The study of transgenerational inheritance in humans is more complex for multiple reasons. One of them is the long time between initial exposure in ancestors and the manifestation of disease in descendants making the discovery of a connection across generations particularly challenging. Furthermore, studies in humans are complicated due to longevity, so observing three generations spans approximately 90 years. In addition to that, unlike previous centuries, the increasing age at which people have children, around 30 years, makes it difficult to gather multiple generations simultaneously. Even if this were possible, this involves follow-up of families and examination of medical records over decades. Few investigations have reported the transgenerational transmission of metabolic and eating disorders in the children of the Leningrad siege survivors (Tolkunova et al., 2023), similarly Holocaust survivor descendants showed transgenerational inheritance of the effects of genocide exposure on the risk and course of schizophrenia (Levine et al., 2016). Many of the parameters examined in humans are complex diseases that are influenced by many factors that accumulate over one's life with incremental effects, making studying the effect of any one single event on a single phenotype difficult (Senaldi and Smith-Raska, 2020).

Additionally, there is a prevalent misuse of the term "transgenerational" in the literature. Many articles incorrectly label their findings as transgenerational in the title, despite the evidence not supporting this classification. For that reason, multiple preclinical models were used to overcome the previous challenging obstacle. One of the first studies that tried to demonstrate non-genomic transmission of traits was conducted by McConnell in 1962 on planaria memory transfer through cannibalism, his experiments involved training planarian flatworms to associate a specific stimulus with an aversive response (such as light with electric shock) and then feeding these trained worms to other untrained worms (Review; Rilling, 1996). However, two years later Karttry and collaborators could not replicate McConnell's work. However, they suggest the existence of an alternative theory (Kartry et al.,

1964). More recent studies validated the transmission of early life experiments through different ELS models. Indeed in 2023 a study proved that stress is transgenerationally transmitted across generations and induces inflammatory and endocrine markers in the uterine tissues of both mothers and their offspring (Lopes et al., 2023). A preclinical model using gestational GCs treated guinea pigs showed altered behaviors, cortisol response to stress and genes expression in the prefrontal cortex and hypothalamic paraventricular nucleus (PVN) up to F3 generation, occurring through both parental lines (Moisiadis et al., 2017). Furthermore in the same mice model, postnatal stress induced behavioral and metabolic changes that persist up to the 5th generation but diminish by the 6th (Boscardin et al., 2022). Another investigation using a mouse model of maternal separation combined with unpredicted maternal stress, reported a transgenerational transmission of symptoms that are consistent and reproducible, and persist with similar severity across generations, including behavioral and metabolic phenotypes up to the 4th generation and depressive-like behaviors until the 3rd generation, and risk-taking and glucose dysregulation until the 4th generation, (van Steenwyk et al., 2018). However, sometimes some studies focus more in the maternal line but their is still evident of transgenerational inheritance through the paternal line, as an example Isabelle Mensuy's lab showed a paternal transmission of behavioral and metabolic traits induced by postnatal stress to the 5th generation in mice (Boscardin et al., 2022). These findings were also observed by Moisiadis and collaborators , indeed transgenerational programming to F3 following antenatal synthetic glucocorticoid, the transmission was sex- and generation-dependent, occurring through both parental lines (Moisiadis et al., 2017).

**Table 1. Studies that characterized both intergenerational or transgenerational transmission of ELS in humans and rodents, showing the clear gap and lack of interventions to correct the inheritance of ELS.**

| Category | Description | Species | N° Generations | Intervention | Source |
|---|---|---|---|---|---|
| Intergenerational transmission | Children and grandchildren of depressed mothers show increased stress response | Human | 2 | No | (Mychasiuk et al., 2011) |
| | Children of parents with PTSD exhibit higher levels of anxiety and depression | Human | 1 | No | (Yehuda et al., 2001) |
| | Offspring show increased stress response and altered behavior | Rats | 1 | No | (Francis et al., 1999) |
| | Prenatal undernutrition impacts offspring's metabolic and cardiovascular health | Rats | 2 | No | (Drake and Walker, |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 2004) |
| | Prenatal stress affects offspring's HPA axis function and behavior | Mice | 1 | No | (Mueller and Bale, 2008) |
| Transgenerational transmission | Maternal stress impacts offspring's stress regulation and behavior | Human | 2 | No | (Painter et al., 2008) |
| | Descendants of Holocaust survivors show altered stress response | Human | 2 | No | (Yehuda and Bierer, 2007) |
| | Second-generation offspring of prenatally stressed rats show altered behavior | Rats | 3 | No | (Skinner et al., 2010) |
| | Prenatal stress effects transmitted to subsequent generations | Rats | 2 | No | (Bertram et al., 2008) |
| | Stress experienced by male mice leads to altered stress responses in offspring | Mice | 2 | No | (Rodgers et al., 2015) |

*Finally, it is evident that early-life stress leaves a lasting blueprint through intergenerational and transgenerational transmission, as demonstrated in* ***Table 1*** *by preclinical and clinical studies. However, there is a gap in interventional approaches, as scientists have prioritized the characterization of these inheritances over intervention development. ELS studies using the perinatal stress model in rats showed the reversal of stress deficits in the offspring of stressed dams when postpartum interventions occurred at the beginning of lactation, such as carbetocin treatments (Gatta et al., 2018; Morley-Fletcher et al., 2024) and adoption (Maccari et al., 1995). However these studies did not investigate further generations other than the direct offspring. Translating these findings into clinical applications presents a complex challenge that requires understanding the mechanisms underlying these transmission effects to develop effective interventions aimed at mitigating these inherited impacts across generations. This complexity arises from the intricate interplay of genetic, epigenetic, and environmental factors, and more importantly the importance of maternal factors in the context of early-life stress. Therefore, future research efforts must focus on elucidating these mechanisms for effective preventive and therapeutic strategies.*

## III. Maternal-mediated factors

After introducing ELS programming through multiple factors, more importantly parental programming via adversities such as nutritional stress, PTSD, war exposure, and

parent-offspring separation, scientists refined their perspectives on programming and more importantly maternal-mediated factors. ELS studies showed the importance of parental deficits and more precisely the maternal component across multiple generations is further underscored by the progress in epidemiology, neurosciences, and related scientific fields, which have identified the perinatal period (comprising prenatal and postnatal phases) as pivotal for health, often seen as a vulnerability window. Seymour Levine's seminal studies in rodents have illuminated critical periods during which environmental factors leave a lasting imprint on the brain (Levine, 1957). Exposure to programming factors during these critical periods can lead to significant and enduring consequences. This vulnerability window can differ from one species to another, depending on their stages of development, and the timing of the adverse factors. Various factors can influence developmental programming, including lifestyle, diet, and pollution. This programming is likely mediated by multiple mechanisms (Sze and Brunton, 2024). However, in the current study, the main factor that we wanted to put at the center of our investigation is *maternal behavior*, but behavior is a complex phenotype, and it's a translation of different factors, from genetic, and hormonal to phenotypic mixture, so to understand better how adversity can affect maternal behavior and how this behavior itself can impact later on the progeny, we should also understand two main factors related to it, the endocrine component that includes stress and anti-stress balance, the *maternal glucocorticoids* (corticosterone) for stress, and *maternal oxytocin* for the anti-stress. Nevertheless, other maternal factors can interfere such as *maternal microbiota*, a new therapeutic avenue in the neuroscientific field for the evident crosstalk between the gut and the brain (gut-brain axis).

## 1. Maternal behavior

### A. Definition

Behavior in general is a complex phenotype. According to psychologists Skinner and Hebb, behavior involves "all observable processes by which an animal responds to perceived changes in the internal state of its body or in the external world" (Jensen, 2013). A key and consistent behavior examined in this PhD is maternal care, which encompasses the actions taken by the dam to nourish and protect her litter during its early stages of development (Orso et al., 2019). In 1990, David Barker famously stated, "The womb may be more important

than the home."(Barker, 1990). Later, scientific reports described the importance of life programming outside the womb such as the *postpartum* period. This perspective has been supported by numerous preclinical and clinical studies over the years, highlighting the critical role of maternal behavior in life programming.

Disruptions in maternal care during early life stages have been identified as major risk factors for the development of psychiatric disorders (Sanson and Bosch, 2022). It's not only the quantity but also the quality of maternal behavior that is crucial. For instance, Toepfer and Ramo-Fernández showed in humans that maternal behavior, characterized by maltreatment, aggression and intrusiveness, can significantly impact maternal and offspring DNA methylation of the oxytocin receptor gene, both in the blood and immune cells (Ramo-Fernández et al., 2021; Toepfer et al., 2019). On the other hand, in rodents, maternal aggression refers to the mother's defensive behavior against potential threats to her offspring, not aggression towards them, which is suppressed in lactating mothers. Therefore, specifically in rodents maternal aggression is considered a positive form of aggression (Bosch, 2013). This reinforces the idea that both positive and negative aspects of maternal care play pivotal roles in shaping long-term outcomes for offspring.

### B. History of maternal behavior studies

Maternal behavior, also referred to as maternal care, has a rich history, tracing back to the early 20th century when researchers began systematically observing and documenting the behaviors of mother animals toward their offspring. In 1950, Harry Harlow conducted legendary and influential studies on Rhesus monkeys, demonstrating the crucial role of maternal contact in the emotional and social development of infants. Harlow's experiments showed that infant monkeys preferred spending time with a soft, cloth-covered surrogate mother over a wire mother that provided food, highlighting the importance of comfort and emotional support in maternal behavior. This work highlighted in his article 'The nature of love' (Harlow, 1958) is without doubt one of the classics of psychology's history (Harlow and Zimmermann, 1959; van Rosmalen et al., 2020). After the outbreak of World War II and its effects on children's psychological wellbeing, in 1950, John Bowlby a child psychiatrist and psychoanalyst was appointed World Health Organization (WHO) consultant to study the needs of children who were impacted by war and needed care in foster homes (van der Horst

et al., 2020). Few years later Bowlby visited Harlow's lab which led to the emergence of the ***attachment theory,*** one of the pioneering works in the psychology field, which laid the foundation for understanding maternal behavior through the attachment perspective. Bowlby's work emphasized the importance of the mother-child bond in the healthy psychological development of children (Bowlby, 1969), this theory is complementary to Barker's DOHaD theory (Barker, 1995a). Both theories underscore the crucial role of early-life experiences in shaping long-term outcomes. The attachment theory emphasizes the impact of early emotional bonds on psychological development, while the DOHaD theory focuses on how early environmental exposures influence physical health. Together, they reinforce the importance of early-life programming on both emotional and health dimensions. By the late 20th century, researchers like Michael Meaney and Moshe Szyf began to uncover the biological underpinnings of maternal behavior through studies on rodents. Their research demonstrated how variations in maternal care, such as licking and grooming in rats, could affect the stress responses and behavioral outcomes of the offspring. This work revealed epigenetic implication in long-term health outcomes (Meaney, 2001; Szyf, 2022; Weaver et al., 2004). Additionally, studies on humans, such as those by Mary Ainsworth, expanded on Bowlby's attachment theory by developing the "Strange Situation" protocol to systematically assess attachment styles in children between the ages of 12 and 18 months. The protocol includes the child being introduced to a new environment, interactions with a stranger, and separations and reunions with their caregiver. Ainsworth results revealed that children can be categorized into secure, insecure-avoidant, insecure-resistant, or disorganized, reflecting the quality of their emotional bond and the caregiver's responsiveness. Thus, this work provided empirical evidence for the impact of maternal behavior on child development (Ainsworth et al., 1978).

*Overall, the first studies on maternal behavior have laid a comprehensive foundation, bridging observations from animal models to human studies. These pioneering works have underscored the critical role of maternal care in shaping the physical, emotional, and psychological development of offspring across species.*

## C. Animal models for maternal behavior studies

Evaluating the adverse effects of stress on maternal care can be particularly challenging due to the lack of consensus on its clinical definition and also to ethical issues. However, understanding and delineating the nuances of maternal behavior is crucial for elucidating the complexities of how early environmental factors influence developmental trajectories, thus leading to the identification of pharmacological targets. Those environmental factors can include among them different stress issues for example stress in work, natural disasters and ongoing wars in different continents.

To overcome the ethical issues in humans, animal models were developed to address the question of maternal care as a programming factor. A systematic review on animal models in 2019 identified a total of 12 different early life stress protocols from 56 studies (Orso et al., 2019), among them in rodent eight different behaviors were analyzed: licking/grooming; arched-back nursing; blanket-nursing/passive nursing; nest building; contact with pups; harmful/adverse caregiving; no contact; nest exits (Orso et al., 2019). But the previous parameters are not the only primordial factors, the frequency of maternal care can be also lifespan impacting, such as maternal behavior fragmentation during critical periods of development.

Among the various animal models, there are the Perinatal Stress (PRS) model, the Prenatal Social Stress (PSS) model, and the Maternal Separation combined with Unexpected Stress (MSUS) model, among others (Brunton, 2013; Franklin et al., 2010; Maccari et al., 1995). However, analyzing maternal behavior generates vast amounts of data, as it is often assessed post-experimentally through video analysis. To address these methodological issues, some modern studies such as the Automated Maternal Behavior during Early Life in Rodents (AMBER) pipeline was developed for quantifying home-cage maternal and mother-pup interactions using open-source machine learning tools such as DeepLabCut to track key points on rat dams and individual pups (Lapp et al., 2023). Similarly, the BAMBI (Bidirectional Assessment of Maternal Behavior and Infant Interaction) method was introduced to automate the assessment of early-life interactions between maternal behavior and pup vocalization in mouse dam-pup dyads (Winters et al., 2023), providing a comprehensive analysis of these interactions, and improvements over traditional video

analysis techniques (Winters et al., 2022). Furthermore, other methods related to AI (Artificial Intelligence) and machine learning are expected to take place in near future to analyze maternal care and its related psychiatric diseases, this will help scientist to make a step further for the prediction of perinatal disorders, but other machine learning applications include (but are not restricted to) biomarker discovery, risk estimation, correlation assessment, pharmacological treatment prediction, drug screening (Mennickent et al., 2023).

### D. How does maternal behavior shape the offspring?

The early work of Michael Meaney and Moshe Szyf labs, indicated that naturally variable maternal behavior could lead to programming in offspring, in this context there was no specific factor imposed to see this variability, their study model served as an important base that paved the way for several scientific studies (Weaver et al., 2004). On the other hand, to understand how this variability can occur scientists used alternative models, defined as ELS models, that were referred to in the section above. From that several findings emerged, all of them highlighting the fact that stress impacts significantly maternal parameters among them maternal behavior.

To push these investigations further, more experimental paradigms were needed to confirm that maternal care plays a crucial role. For example in 1995, Maccari and collaborators, came with a striking study showing that adoption reversed stress response deficits in offspring descending from stressed dams (Maccari et al., 1995), what was more surprising was the fact that foster mothers spent more time doing maternal behavior compared to biological mothers (Maccari et al., 1995). Maccari's lab took further these finding, and tried a pharmacological approach to correct maternal behavior, via *postpartum* carbetocin treatments, and the results were striking; inducing maternal behavior via an agonist of oxytocin receptors such as carbetocin, increased maternal behavior in stressed dams and led to the reversal of stress deficits in the offspring (Gatta et al., 2018).

Subsequent research has provided deeper insights into the complex interplay between maternal behavior and offspring development, revealing a wealth of interconnected findings. Studies in rats have elucidated the pivotal role of maternal nurturing in regulating neural systems associated with fearfulness expression (Caldji et al., 1998). Maternal separation experiments conducted with Wistar rats have yielded striking revelations, demonstrating that

such separation induces cognitive deficits in adolescent offspring (Alves et al., 2022). Additionally, maternal stress, particularly through the activation of the CRH receptor 1 pathway, has been shown to impair maternal behavior and trigger local oxytocin release in lactating rats. This sheds light on the neurobiological mechanisms underlying maternal stress responses, further emphasizing the intricate interplay between stress physiology and maternal behavior (Klampfl et al., 2018). Chronic psychosocial stress during pregnancy has been identified as another significant factor affecting maternal behavior and neuroendocrine function. Studies have demonstrated its detrimental effects on maternal well-being, further emphasizing the importance of addressing psychosocial stressors during pregnancy (Zoubovsky et al., 2020).

In human populations, prenatal maternal negative life events have been associated with heightened risks of emotional and behavioral problems in children. This underscores the cross-species relevance of maternal stress on offspring well-being and highlights the need for comprehensive support systems for expectant mothers (Avendano et al., 2024). Epigenetic studies have revealed that breastfeeding is linked to decreased DNA methylation of the glucocorticoid receptor promoter, as well as reduced cortisol reactivity in infants. These findings illuminate the epigenetic mechanisms through which maternal behavior shapes stress responses in offspring, underscoring the long-term implications of early maternal interactions (Lester et al., 2018).

*Together, these findings paint a comprehensive picture of the complex relationship between maternal behavior and offspring development, emphasizing the complex and multifaceted nature of maternal influence, highlighting its significant and wide-ranging implications for offspring well-being across species. Furthermore, to better approximate real-life conditions, it is important that methodological approaches for evaluating maternal behavior—such as time, duration, and behavior type become standardized and made more consistent across studies, but in human not all women go through the same stress and react the same to it, or live the event at similar stages of pregnancy or postpartum periods, each human is unique so at the end heterogeneity in stress protocols are not that bad for our understandings, we need just to adapt and find ways to draw conclusion and find windows of comparisons between the preclinical and the clinical.*

## 2. Maternal hormonal environment (the stress/anti-stress balance)

### A. Stress (Glucocorticoids)

In general, in response to stress, the brain activates several neuropeptide-secreting systems. This eventually leads to the release of adrenal corticosteroid hormones (de Kloet et al., 2005). This neuroendocrine response involves the hypothalamic-pituitary-adrenal (HPA) axis, which plays a critical role in regulating stress. For decades, scientists have investigated how these stress hormones, essential for life, can shift from protective to harmful, increasing vulnerability to stress-related disorders, more particularly during sensitive periods such as gestation and *postpartum*.

#### a. The HPA axis

When an external stressor occurs, the hypothalamic neurons (in the PVN) active the release of CRH into the hypophyseal portal plexus of veins, then travel to the anterior pituitary and bind G-protein coupled receptors (corticotropin releasing hormone R1 receptors; CRHR1), thus enhancing transcription of the proopiomelanocortin (POMC) gene, which encodes adrenocorticotropic hormone (ACTH) and leads to its release. Once into the bloodstream, ACTH travels to the adrenal glands and binds to specific receptors on the surface of adrenal cortex cells (Herman et al., 2016). The final products of the HPA axis are cortisol (human) and corticosterone (rats/mice), naturally occurring glucocorticoids produced by the adrenal glands (Herman et al., 2016). These hormones play crucial roles in regulating various physiological processes, including stress response, metabolism, and immune function. The multiple steps of stress response are simplified in **Figure 2**.

Later on investigations started to explore the exact mechanism underlying the HPA axis and its regulation, and it was addressed that the action of glucocorticoids was mediated by two types of corticosteroid receptors; the mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (de Kloet and Meijer, 2019). Both GR and MR are members of the nuclear receptor superfamily and function as transcription factors upon binding to their ligands. GR has a lower affinity for glucocorticoids compared to MR and is primarily involved in the stress response, while MR, with its higher affinity, plays a crucial role in regulating electrolyte balance and basal cortisol levels. Upon binding glucocorticoids, GRs and MRs translocate to the nucleus, where they bind to specific DNA sequences called glucocorticoid

response elements (GREs) as homo- or heterodimers to regulate the transcription of target genes. MR and GR share 96% homology in their DNA binding domain, and this genomic action of GR and MR is critical for modulating the expression of genes involved in metabolism, immune response, and other vital functions during stress (de Kloet and Meijer, 2019).

In summary, the foundational work by Hans Selye and subsequent researchers contributed to the understanding of the complex role of the HPA axis and glucocorticoid signaling in stress and physiological regulation, the full HPA axis is presented in **Figure 2**. Understanding the distinct roles of GR and MR as transcription factors provides deeper insights into how glucocorticoids orchestrate a wide array of bodily responses to maintain homeostasis and respond to stress.

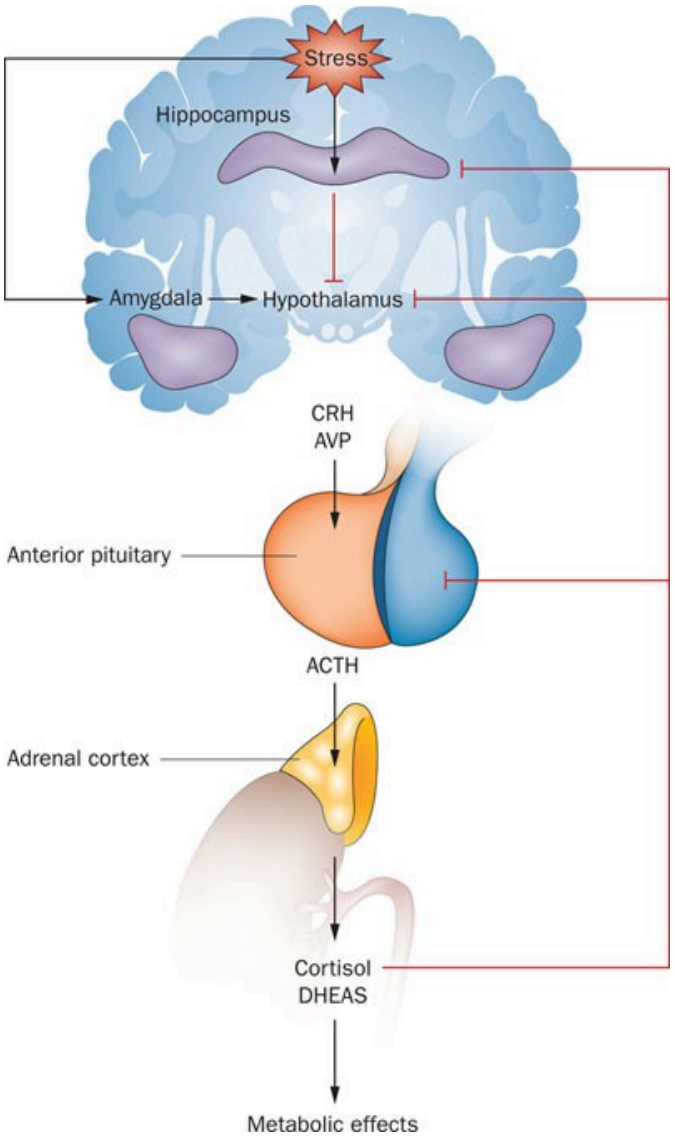


**Figure 2. HPA axis From (Papadopoulos and Cleare, 2012)**.

Additionally, synthetic glucocorticoids such as dexamethasone or betamethasone, designed to mimic the effects of natural glucocorticoids, are widely used in clinical settings due to their potent anti-inflammatory and immunosuppressive properties (Coutinho and Chapman, 2011; Langarizadeh et al., 2021), these compounds are also utilized in preclinical models of early-life stress (ELS) to induce specific experimental conditions. Therefore, their introduction is crucial in the context of understanding ELS and its impact on HPA axis function. De Kloet and colleagues have contributed significantly to understanding the sequential activation of the HPA axis in response to stressors. But detecting GR through *in vivo* radioligand binding studies posed challenges for two main reasons. Firstly, the amount of radiolabeled corticosterone tracer used was insufficient to occupy GR, which binds cortisol

and corticosterone with approximately tenfold lower affinity compared to the high-affinity MR (de Kloet and Meijer, 2019). Secondly, in vivo studies using the high-affinity GR ligand dexamethasone, failed to yield a signal in the brain due to its active export by P-glycoprotein, which is located at the blood-brain barrier and prevent access of glucocorticoids to the brain (de Kloet and Meijer, 2019). Furthermore, this research highlighted differences between GR and MR, underscoring that GR becomes activated under stress conditions with higher glucocorticoid levels (de Kloet and Meijer, 2019). This differential affinity is crucial for regulating the physiological responses mediated by glucocorticoids in various contexts. This led to the characterization of HPA axis activity as a sequential activation process, underscoring the intricate balance and adaptive responses mediated by glucocorticoids.

### b. How maternal glucocorticoids shape the offspring

The brain is particularly sensitive to prenatal influences, especially glucocorticoids, which play a crucial role in early brain development. Research shows that maternal stress can alter uterine artery blood flow, thereby impacting fetal development. Although there is a protective enzyme in the placenta, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) that breaks down cortisol and corticosterone into their inactive forms, some glucocorticoids may still cross to the fetus, influencing its development (de Kloet and Meijer, 2019). Seckl's lab has highlighted that in animal models, the inhibition or knockout of 11β-HSD2 results in offspring with reduced birth weight and causes permanent changes, including hypertension, hyperglycemia, increased HPA axis activity, and behaviors resembling anxiety (Seckl and Holmes, 2007).

Gestational stress during both early pregnancy and/or late pregnancy have negative outcomes. Stress during early pregnancy leads to the activation of the maternal HPA axis which might jeopardize the pregnancy (Brunton et al., 2008). Rodents exposed to stress during implantation show heightened ACTH secretion and increased risk of pregnancy failure due to progesterone secretion inhibition. On the other hand, during late pregnancy HPA axis responses to a wide range of stressors (Brunton et al., 2008). In the perinatal stress model, which combines both a prenatal stress (during gestation, through increased maternal glucocorticoids) and postnatal stress (during *postpartum* period through reduced maternal behavior), showed that PRS offspring present altered HPA axis through prolonged

corticosterone secretion after stress and reduced GR in the hippocampus (Maccari et al., 2003). Furthermore, in the PRS model, gestational stress induced a reduction in placental expression and activity of the enzyme 11β-HSD2 (Mairesse et al., 2007). In a clinical study, the enzyme 11β-HSD2 was found to have mutations and reduced activity in vulnerable populations, such as Jews from Morocco and Iran, who experienced major traumatic and stressful events (Rösler and White, 1993).

Recent investigations showed that maternal glucocorticoids can induce deficits beyond the exposed mother showing long lasting blueprints in the offspring. In mammals, prenatal maternal stress in sheep showed deficits in the offspring including impaired birthweight, HPA axis, behavioral patterns and cognitive abilities and altered gene expression and brain morphology (Wei et al., 2023). Furthermore, same kind of deficits were observed in rodent, a study found that dams exposed to synthetic glucocorticoids (sGC) exhibited altered corticosterone responses to stress, which were transmitted to juvenile females and males, additionally, behavioral deficits were associated with altered gene expression in the prefrontal cortex and hypothalamic paraventricular nucleus (PVN), impacting networks related to type II diabetes, thermoregulation, and collagen formation (Moisiadis et al., 2017). In rats, prenatal stress (PNS) induced hyperactive HPA axis responses and heightened anxiety in adult male offspring, these effects were transmitted to the F2 generation via the maternal line. These effects were sex-dependent, with no differences in depressive-like behavior observed in either sex (Grundwald and Brunton, 2015). Furthermore, a study investigated the effects of low or high levels of CORT administered to dams during gestation, *postpartum*, or both. High CORT during *postpartum* increased depressive-like behavior in dams and altered maternal care regardless of gestational timing. Both high CORT treatments reduced hippocampal cell proliferation in *postpartum* dams compared to controls (Brummelte and Galea, 2010). In rat model, treatments using steroid hormones related to stress (Glucocorticoid) induced loss of DNA methylation in nonneuronal cells involving DNA methyltransferase 1 (DNMT1) in epigenetic regulation, evidencing that stress can directly impact epigenetic and more specifically DNA methylation mechanisms (Yang et al., 2012). Furthermore, offspring from mothers with high fecal glucocorticoid metabolite (FGM ) levels experienced lower survival rates and received less care (Pinho et al., 2019). Interestingly, under adverse conditions, pups from mothers with high FGM levels had higher survival rates when born in small litters

(Pinho et al., 2019). Additionally, in humans preterm birth and maternal educational status were associated with differences in glucocorticoid concentrations, while no significant differences were found between exclusive and partial breastfeeding women (Pundir et al., 2019).

*In summary, stress during early life, when development is still incomplete, appears to have a significant impact on health in both dams and offspring, specifically when two major protective barriers are shut down as reduced HPA axis feedback and altered 11β-HSD2 activity. Since the identification of the stress axis in 1936, scientists have been "digging gold." Knowing the exact locations of MR and GR in the brain, and implicated enzymes paved the way to new perspectives for understanding stress and its molecular, cellular, neuroendocrine, and behavioral functions.*

## B. Anti-Stress (oxytocin)

Nature is perfectly done because when there is stress, there is possibly something called anti-stress to down-regulate stress response beyond the natural negative loop (GR and MR negative feedback). Among the various anti-stress candidates, oxytocin is a well-studied neuropeptide known for its effects on behavior and physiological balance. This neuropeptide has attracted great attention from neuroscientists, psychiatrists, and psychologists for its effect on social behaviors, anxiolytic, and potential application for the treatment of mental diseases associated with altered social competence (Grinevich and Neumann, 2021).

### a. Oxytocin history and structure

In 1953, Vincent du Vigneaud determined the chemical composition of oxytocin and it became the first peptide hormone to have its sequence of amino acids identified, two years later, Du Vigneaud was awarded the Nobel Prize in Chemistry 1955 (Du Vigneaud et al., 1953). Over 50 years, oxytocin earned the nickname "Love hormone" as its association with love became firmly established through scientific studies and media coverage by the mid-2000s. Thanks to Du Vigneaud's work, it is well known now that the small peptide hormone contains nine amino acids arranged cyclically, the structure is stabilized by a cysteine sulfhydryl bridge between amino acids 1 and 7 (Ivell et al., 2018). This unique structure enables resistance to rapid degradation in the bloodstream and facilitates precise interaction with its receptor (OTR; Oxytocin Receptor) and binding protein (Neurophysin 1).

Moreover, some of the wide range functions of oxytocin may be explained by the dynamic biological properties of the sulfur bonds that create the ring in oxytocin and that allow the oxytocin molecule to form temporary and long-lasting unions with other chemical entities such as TrkB, estrogen and vasopressin receptors (Carter, 2014; Mitre et al., 2022; Murakami et al., 2011). In mammals, oxytocin and vasopressin evolved from a common ancestral hormone found in invertebrates like annetocin in worms. Originally, these hormones served basic functions such as muscle contraction and osmotic balance (Ivell et al., 2018). In modern mammals, oxytocin mainly controls contractions in the uterus and vas deferens, while vasopressin regulates kidney function for osmotic balance. Through evolution, both hormones have developed new roles, leading to complex regulation and varied patterns of expression (Ivell et al., 2018).

### b. Oxytocin synthesis and mode of action

Oxytocin is synthesized in the hypothalamus by neurons (magnocellular cells and parvocellular cells) in the supraoptic (SON) and paraventricular (PVN) nuclei. These nuclei have axons that project to the posterior pituitary, where oxytocin is stored for peripheral release, more precisely, magnocellular cells mainly project to the posterior pituitary glands and release oxytocin in the bloodstream to influence peripheral functions as a neuronal hormone, while parvocellular cells project to the midbrain and the spinal cord to control autonomic functions.There is two different types of release, including direct axonal release and dendritic release **Figure 3A** (Carter, 2014; Liao et al., 2020; Matsuzaki et al., 2012). The hypothalamic neuropeptide target mainly the oxytocin receptor (OTR), part of the G protein-coupled receptor (GPCR) family, it is extensively expressed throughout the central nervous system (CNS), with notable concentrations in the ventromedial nucleus of the hypothalamus, the central nucleus of the amygdala, the hippocampus and many other brain regions in the CNS (e.g. prefrontal cortex regions, striatum,..) as evidenced in the **Figure 3B** (Matsuzaki et al., 2012; Szafoni and Piegza, 2022).

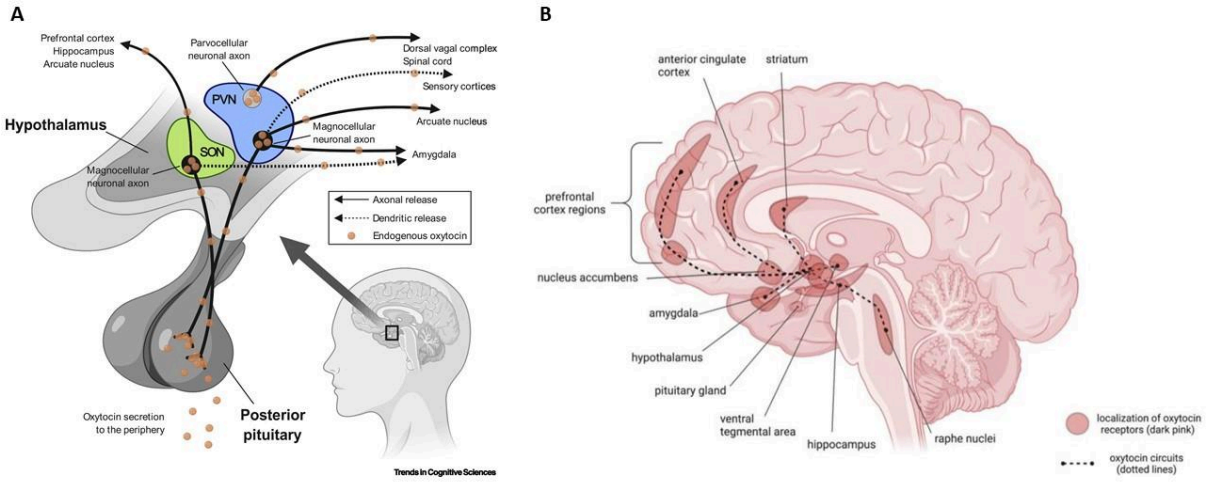


**Figure 3. A) Oxytocin synthesis pathways (Quintana and Guastella, 2020). B) Localization of oxytocin receptors (dark pink) and oxytocin circuits (dotted lines) (Szafoni and Piegza, 2022).**

In a groundbreaking article by Grinevich and colleagues, the integration of techniques such as in situ hybridization, transcriptomic analysis, and autoradiography has provided a comprehensive view of OT and OTR ontogeny in the brain throughout development (Grinevich et al., 2015; Grinevich and Neumann, 2021). Specifically, the rat, a well-studied mammalian species, exhibits a documented developmental trajectory of OTR expression from embryonic stages through adulthood. During early development, OTR emerges in specific brain regions, establishing an "infant" pattern by postnatal day 10 (PN10). Subsequent developmental phases around PN13 and PN18 signify transitions to an adult pattern characterized by dynamic changes in OTR density across various brain regions. Another notable transition occurs around weaning (P60-90), further reshaping OTR expression **Figure 4A**. Key brain structures implicated in these developmental phases include the anterior olfactory nucleus, caudate putamen, accumbens, cingulate cortex, and hypothalamic nuclei **Figure 4B** (Grinevich et al., 2015; Grinevich and Neumann, 2021).

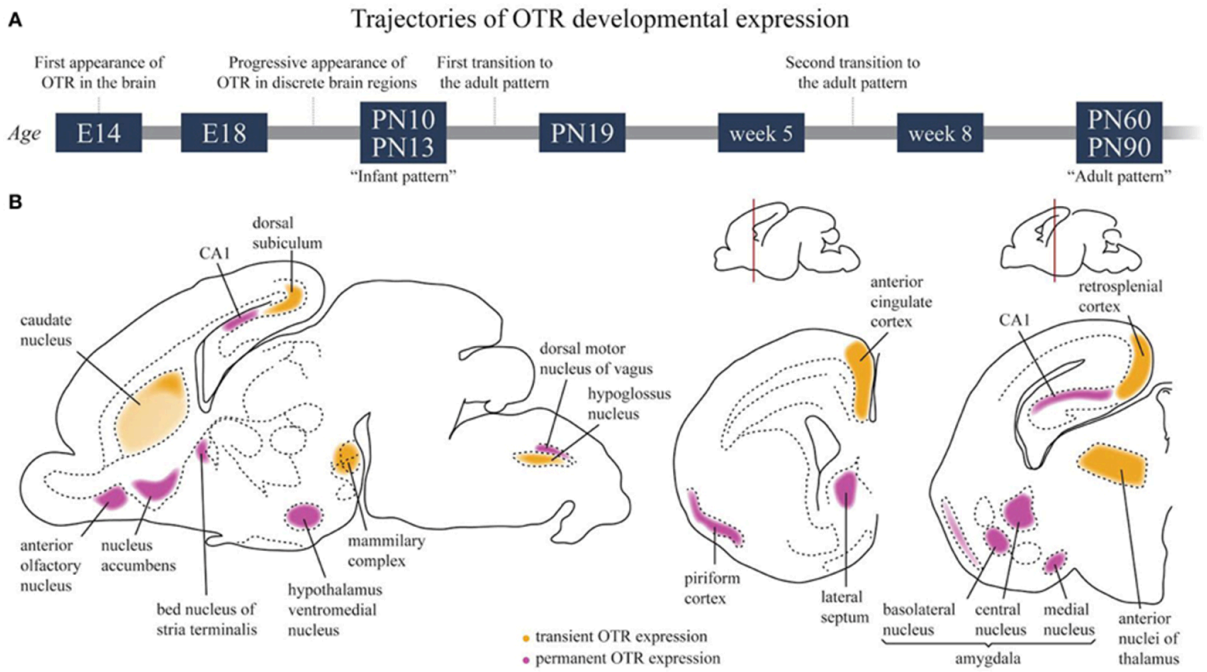


**Figure 4. A) Schematic time course of OTR expression in the developing brain. B) OTR expression in the infant brain around P10–P13.** Regions in which a transient OTR expression is observed are colored in yellow; regions in which OTR expression is maintained to adult life are colored in magenta (Grinevich et al., 2015).

Due to its widespread distribution, OT functions as a neurotransmitter and neuromodulator, influencing a wide range of CNS activities in both males and females. These functions encompass emotional regulation, parental behaviors , affiliative interactions , and sexual behaviors (Matsuzaki et al., 2012). The actions of OT produced by neurons in the SON and the PVN of the hypothalamus are distinct due to their differing roles and projections. The first, is primarily involved in the regulation of osmotic balance. Neurons in the SON project mainly to the posterior pituitary, where oxytocin is released into the bloodstream. This oxytocin plays a crucial role in milk ejection during lactation (Russell and Douglas, 2003). The OT produced in the PVN, has broader and more diverse roles, including stress response, metabolic regulation, and social behaviors. Neurons project not only to the posterior pituitary but also to other brain regions and the spinal cord. This intricate neural network influences autonomic functions and integrates neuroendocrine responses, including the HPA activity (Jiang et al., 2023; Jurek and Neumann, 2018) OT exerts its wide activity through its GPCR receptor OTR (Jiang et al., 2023; Jurek and Neumann, 2018). The OTR, characterized by seven transmembrane domains, is specifically activated by OT, playing a pivotal role in various physiological and behavioral processes. OTR is prominently expressed in the hypothalamus, hippocampus, pituitary gland, adrenal gland, and immune elements such as

microglia, astrocytes, and the thymus. This receptor facilitates the effects of OT across diverse bodily functions, including the formation and regulation of social bonds (Jiang et al., 2023; Jurek and Neumann, 2018). Moreover, OTR undergoes glycosylation in its N-terminal region, a post-translational modification crucial for its proper folding, stability, and interaction with OT ligands (Wang et al., 2020). This wide expression and glycosylation underline OTR's significance in facilitating the effects of OT across diverse bodily functions, particularly in the formation and regulation of social bonds. **Figure 5** summarizes the structure of the ligand OT and its receptor OTR.

In summary, the now well-established capacity of OT to play a role in social bonds appears to be built upon the chemistry of this remarkable molecule, which itself forms bonds throughout the body and this phenomenon is also reliant on its widely expressed receptor; the OTR.

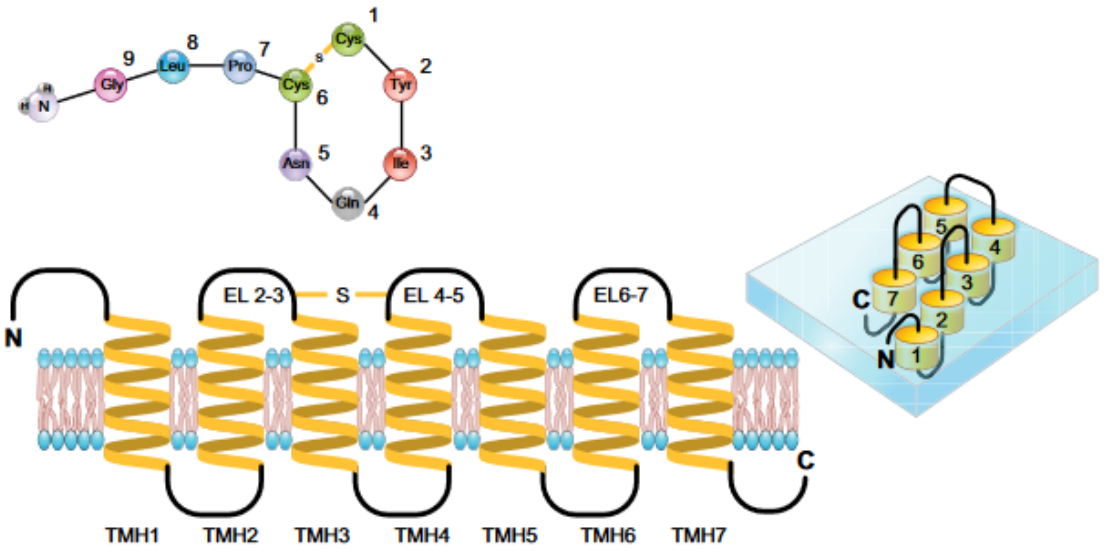


**Figure 5. Structure OT and OTR**, OT consists of a linear tripeptide with an amidated COOH terminus and a cyclic structure connected by a disulfide bridge between two cysteines. OTR Structure: OTR is a seven-transmembrane helix receptor (TMH1–7) with three extracellular loops (2–7) and three intracellular loops (1–6). The receptor undergoes glycosylation, particularly in its N-terminal region, which can affect its folding, stability, and interaction with ligands. The binding pocket for the OTR antagonist d(CH2)5[Tyr(Me)2,Thr4,Orn8,Tyr9]-vasotocin is located between TMH 1, 2, and 7. The OT binding site includes the NH2-terminal region and extracellular loops 2–3 and 4–5 (Jurek and Neumann, 2018).

### c. How maternal oxytocin shapes the offspring

Since Meisenberg's first report in 1981, which demonstrated the pivotal role of oxytocin (OT) in mouse behavior such as grooming and scratching via the anterior pituitary (Meisenberg, 1981), significant progress has been made in understanding OT's role in maternal brain

adaptations. Around birth, behavioral and physiological changes in the maternal brain ensure reproductive functions, maternal care, and offspring survival. These adaptations include reduced hormonal stress responses and anxiety-related behavior, leading to general calmness. Increased activity of inhibitory systems like oxytocin and prolactin, and reduced activity of excitatory pathways, contribute to these changes (Slattery and Neumann, 2008). Indeed, naturally, it has been shown in various mammalian species that the responsiveness of the HPA axis to a broad variety of psychological and physiological stressors is severely attenuated from mid-pregnancy until the end of lactation (Brunton et al., 2008). These maternal stress adaptations are essential for healthy prenatal development, postnatal maternal behavior, and the mental health of the mother and offspring (Slattery and Neumann, 2008). Indeed, in mammals, in order to prepare the mother for the birth, during pregnancy both physiological and behavioral changes occur through the oxytocinergic system; these changes include the onset of maternal behavior, milk production and nursing of offspring (Slattery and Neumann, 2008).

Recent animal studies have shown that OT triggers the initiation of maternal behavior in *postpartum* rats within the ventral tegmental (midbrain) and medial preoptic (hypothalamus) areas (Pedersen et al., 2011). Furthermore, Robert C. Froemke lab, a pioneer team working on OT gave further evidence of the importance of maternal OT in early life programming. Indeed, Marlin and collaborators showed that OT enables pup retrieval behavior in female mice by enhancing auditory cortical pup call responses, and accelerated retrieval behavior through OT wich required only left auditory cortex, through OTR, thus giving first evidence of a lateralized neural responses to pup calls (Marlin et al., 2015). In 2016, the Froemke lab developed a specific OTR antibody for mice and investigated its expression across the brain. This research revealed a maternal behavior network, enriched with OTRs, distributed across various brain regions, including the piriform cortex, the left auditory cortex, and CA2 of the hippocampus (Mitre et al., 2016). Further examination of the cerebral cortex indicated that OTRs were predominantly located at synapses, as well as on axons and glial processes. Additionally, OT temporarily decreased synaptic inhibition in various brain regions and facilitated long-term synaptic plasticity in the auditory cortex (Mitre et al., 2016). Specifically, in a chronic stress condition, OT enables maternal behavior by sensitizing the cortex, surprisingly both male and female mice showed parental behavior after cohousing

with experienced females (Mitre et al., 2017). However, OT treatment accelerated the onset of parental behavior in females but not in males, indicating that the left lateralization of OTR expression in females offers a unique mechanism for accelerating the initiation of maternal behavior. Although male mice can also co-parent effectively after exposure to infants, the sex-specific pattern of OTR expression may genetically predispose the female cortex to be more responsive to infant cues, a response that both males and females can quickly learn (Mitre et al., 2017). However, in vivo studies are still needed to fully elucidate how oxytocin (OT) enhances maternal behavior toward pups during the *postpartum* period. More recently, a study found that OT neurons specifically responded to pup vocalizations, but not to pure tones, through input from the posterior intralaminar thalamus. Additionally, repetitive thalamic stimulation induced lasting disinhibition of OT neurons, providing a mechanism for integrating sensory cues from offspring into maternal endocrine networks. This integration ensures modulation of the brain state for efficient parenting (Valtcheva et al., 2023). However, the literature still presents some contradictory findings. For instance, maternal separation in Kyoto rat dams unexpectedly led to increased pup-contact, which elevated OT levels and reduced anxiety-like behavior after weaning. Nevertheless, this separation also negatively impacted the cognitive behavior of their adolescent offspring (Alves et al., 2022).

Taken together, it is evident that OT plays a crucial role in maternal care, as highlighted in preclinical models. In clinical studies, the closest equivalent observations are during lactation period, particularly breastfeeding. Indeed, consistent with animal studies, late human pregnancy and lactation are associated with increased calmness, a more positive mood state, and a reduced emotional response to stressful life events (Carter et al., 2001). Another early study in humans suggested that these changes may be due to a reduced activity of the CRH system (Slattery and Neumann, 2008), and suckling-related factors, including the activation of the brain OT systems, were shown to contribute to the reduced HPA axis response to stress and to the positive mood state (Heinrichs et al., 2001). More recently, a study assessing breastfeeding complications and maternal mood at 8 weeks *postpartum* found that breastfeeding difficulties, whether alone or alongside physical problems, were associated with poorer maternal mood (Cooklin et al., 2018). This was concordant with Hamdan and Tamim findings (Hamdan and Tamim, 2012), in a prospective study, it was shown that breastfeeding mothers had lower scores on the Edinburgh Postnatal Depression Scale (EPDS)

at 2 and 4 months *postpartum* and were less likely to be diagnosed with *postpartum* depression at 4 months. Additionally, the study revealed that higher depression scores at 2 months *postpartum* predicted lower breastfeeding rates at 4 months. Another prospective study found a significant decrease in depression scores from the third trimester of pregnancy to 3 months *postpartum* in mothers who exclusively breastfed for more than 3 months, compared to those who breastfed for less than 3 months (Krol and Grossmann, 2018). Investigations into the potential mechanisms revealed that, in humans, low maternal care during childhood was associated with increased DNA methylation in the OTR and BDNF sequences in blood cells at adulthood. Despite limitations such as small effect sizes and uncertainty regarding the relevance of changes in blood cell gene methylation to brain gene methylation, the findings still suggest elements of an epiphenotype resulting from early life stress (Unternaehrer et al., 2015).

### d. The anti-stress effect of the oxytocinergic activation as a therapeutic avenue

In 2024, scientific interest in OT surged, resulting in nearly 31,000 publications that advanced our understanding of this hormone and its role in various physiological processes. Oxytocin-producing cells and their receptors (OTR) are responsive to the OT peptide, and under certain conditions, autocrine feedback mechanisms regulate their function. Early-life stimulation of the OT system can enhance its activity, leading to increased OT release (Carter et al., 2020; Kenkel et al., 2019, 2014). These conditions were reviewed in a comprehensive study by Nagasawa, highlighting that social interactions such as parenting and mating activate the oxytocin system. This enhances individual parenting behavior towards infants and fosters a positive cycle of oxytocin and caregiving. This interaction promotes attachment between parents and infants, influencing behaviors across generations (Nagasawa et al., 2012). This positif loop mechanism presents a promising therapeutic opportunity for addressing early-life stress. If scientists can harness endogenous OT production, it could offer hope for treating psychiatric disorders. Furthermore, the influence of intraperitoneal injections (IP) of OT in early life extends into adulthood by regulating the expression of OTR (Kenkel et al., 2019). The availability of OTRs can also be modulated through the administration of exogenous OT (Robinson et al., 2003; Smith et al., 2006). Overall, this feedback system represents a significant therapeutic avenue aiming to mitigate stress.

Oxytocin, commonly used during labor especially in the United States, has potential long-term developmental effects on offspring that are not fully understood. A study using prairie voles found that maternal OT administration at birth increased methylation of the OTR gene in the fetal brain (Kenkel et al., 2019), Additionally, this comprehensive investigation underscored that maternal OT IP at birth influences offspring behavior across development, impacting social behavior and the endogenous oxytocin system (Kenkel et al., 2019). A pioneering work by Kabbaj's lab, demonstrated that histone deacetylase inhibitors promote partner preference formation in prairie voles through epigenetic mechanisms, and involves enhanced expression of OTR and AVP receptors in the nucleus accumbens, underscoring the role of epigenetics in regulating social bonding (Wang et al., 2013). Adult voles exposed to OT showed increased social behaviors, such as alloparental caregiving and close social contact. These changes were attributed to direct effects on the offspring rather than changes in maternal behavior, with male offspring showing increased OTR density and expression in the brain (Kenkel et al., 2019). This suggests that perinatal OT exposure may exert long-term effects through epigenetic mechanisms.

In addition to these findings, intranasal (IN) OT administration appears as a new promising route for human and preclinical studies. Indeed, intranasal administration is described in some reports as a "direct entry into the brain" (Quintana et al., 2021). Following intranasal administration, elevated levels of OT have been observed in the cerebrospinal fluid (CSF) of both rhesus macaques and humans. Although this does not definitively prove that the neuropeptides have directly reached the brain, a growing body of evidence suggests they likely do so through the olfactory and trigeminal nerve pathways (Quintana et al., 2021). To solidify the context of this study, reports in humans using intranasal OT have been demonstrated to enhance the stress-protective effects of social support in women with negative childhood experiences during a virtual Trier Social Stress Test (Riem et al., 2020). In preclinical rat models, intranasal oxytocin attenuates stress responses following chronic complicated stress (Yang et al., 2019), and exerts anti-aggressive and pro-affiliative effects in male rats (Calcagnoli et al., 2015).These findings highlight the potential of intranasal oxytocin as a practical and effective approach for mitigating stress deficits, making it a promising candidate for treating stress-related disorders.

Furthermore, *postpartum* treatment with analog of OT (CBT: carbetocin), corrected maternal behavior that was reduced in stressed dams, and through maternal care CBT had epigenetic correction in stress-related genes, thus showing a disease-dependent effect by correcting HPA axis activity and risk-taking behavior in the offspring (Gatta et al., 2018). This therapeutic strategy also reversed the metabolite alterations associated with long-term cognitive and emotional alterations alongside endocrinological disturbances (Morley-Fletcher et al., 2024). Indeed, carbetocin is more stable than oxytocin since the half-life of CBT is 40 minutes, which is 4-10-fold longer than that of OT (Jin et al., 2019), which explains why a single dose of carbetocin appears to be more effective than oxytocin for several hours (Fahmy et al., 2016).

*The relationship between hormonal balance, specifically stress (GCs) and anti-stress (OT) deficits, and psychiatric disorders is complex. Although multiple studies have investigated this relationship, few have explored the therapeutic potential of OT's anti-stress capacity in treating these disorders. Further research is needed to fully understand the underlying mechanisms and to evaluate the therapeutic implications of targeting both systems. Additionally, individual variability and other factors may influence disease susceptibility and progression.*

### C. Brain-Derived Neurotrophic Factor (BDNF): The potential mechanism in the brain

Deficient maternal behavior and imbalances in stress regulation profoundly affect molecular biomarkers in the brain. One such critical molecule is BDNF (Brain-Derived Neurotrophic Factor), which belongs to the neurotrophin family of growth factors. Unlike other proteins produced by the brain that circulate systemically, BDNF acts locally within the nervous system. It plays a pivotal role in synaptic plasticity, neuronal growth, and neuroprotection, essential for the survival, development, and optimal functioning of neurons in both the central and peripheral nervous systems.

#### a. BDNF gene

BDNF gene is very complex composed of various exons controlled by multiple promoters. In the early 2000s, the only available data on the structure and regulation of the BDNF gene came from Timmusk and colleagues, who identified four 5’ exons connected to distinct

promoters and one 3' exon encoding the preproBDNF protein in rats (Pruunsild et al., 2007). Later on, other investigations revealed the presence of at least nine exons (Notaras and van den Buuse, 2019). **Figure 6** illustrates BDNF gene in both rodents and humans.

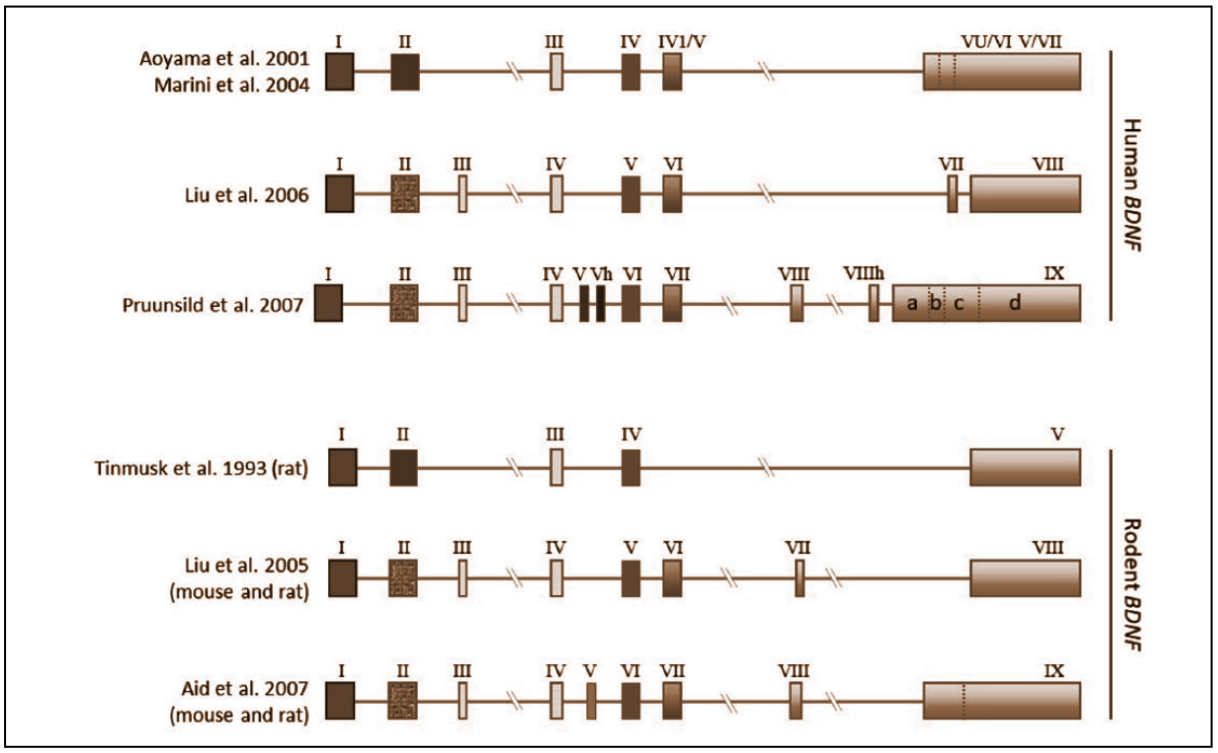


**Figure 6. Genomic structure of both the human BDNF and rodent Bdnf genes**. Exons are colored according to between-study and between-species analogy. Exons are shown as boxes whereas introns are shown as lines (Notaras and van den Buuse, 2019).

### b. BDNF protein and its isoforms

***BDNF*** is a highly conserved protein from the neurotrophin family, essential for the survival, growth, and maintenance of neurons. BDNF is synthesized in the brain, particularly in regions such as the hippocampus, cortex, and basal forebrain. Among its key functions are the regulation of neuronal and glial development, neuroprotection, and the modulation of both short- and long-term synaptic interactions, all of which are essential for cognition and memory (Kowiański et al., 2018). A wide spectrum of processes are controlled by BDNF, and the sometimes contradictory effects of its action can be explained based on its specific pattern of synthesis, comprising several intermediate biologically active isoforms that bind to different types of receptor, triggering several signaling pathways (Kowiański et al., 2018). BDNF is initially produced as a precursor protein known as proBDNF. The conversion of proBDNF to mature BDNF involves cleavage by specific proteases, a process crucial for the

appropriate regulation of BDNF's functions in the brain (**Figure 7A**). BDNF also undergoes post-translational modifications, including glycosylation and cleavage by proteases such as furin and prohormone convertases, to produce the mature form of BDNF. This processing can occur intracellularly within the endoplasmic reticulum and Golgi apparatus, as well as extracellularly by enzymes like plasmin and matrix metalloproteinases (Foltran and Diaz, 2016). BDNF transmits signals via the tropomyosin receptor kinase B (TrkB) and the low-affinity p75 neurotrophin receptor (p75NTR) (Colucci-D'Amato et al., 2020)**.** ***ProBDNF*** is the precursor form of BDNF, which is initially synthesized as a longer pro-peptide that includes the mature BDNF sequence. What is intriguing about proBDNF is that it can bind to receptors and has distinct biological activities compared to mature form. It is primarily associated with apoptosis (programmed cell death) and can negatively regulate synaptic strength and plasticity (**Figure 7B**)(Yang et al., 2014).

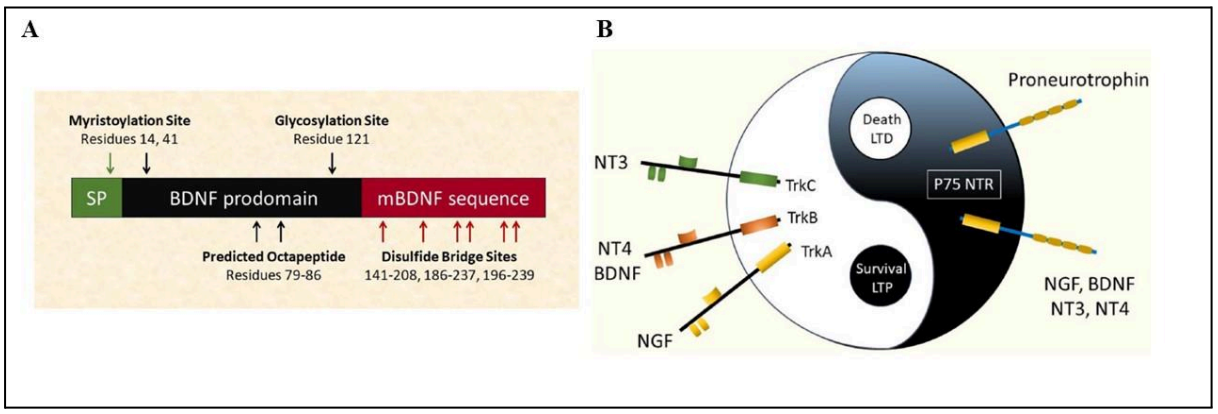


**Figure 7. Cleavage and role of BDNF** A) BDNF Structure consists of a signal peptide (amino acids 1-18), a low complexity region (amino acids 100-111), and a nerve growth factor (NGF) family domain (amino acids 133-246) following cleavage at residue 129. Known and predicted posttranslational modification (PTM) sites are indicated. B) Yin and Yang of BDNF: Mature neurotrophins bind strongly to their receptors, promoting neuronal survival, growth, and long-term potentiation (LTP). In contrast, pro-neurotrophins and mature neurotrophins with lower affinity can induce cell death and long-term synaptic depression (LTD) by binding to the p75 neurotrophin receptor (p75NTR). NGF, NT3, and NT4 were not discussed in this study. Figure adapted from (Notaras and van den Buuse, 2019).

Indeed, cleavage-resistant proBDNF mice showed reduced hippocampal dendritic complexity and spine density due to p75(NTR) (Yang et al., 2014),and hippocampal slices from proBDNF mice show reduced synaptic transmission, impaired LTP, and increased LTD in CA1, indicating that proBDNF regulates hippocampal structure, synaptic function, and plasticity differently from mature BDNF (Yang et al., 2014). On the other hand, in rodents proBDNF knockout causes abnormal motor behaviors and leads to early death in mice (Li et al., 2020). The binding complex of proBDNF, p75NTR, and sortilin triggers signaling

cascades that activate c-Jun N-terminal kinase (JNK), a pathway implicated in neuronal apoptosis (Teng et al., 2005). ***Truncated BDNF*** refers to shorter forms of proBDNF that result from alternative splicing or proteolytic processing. These forms can have different biological functions compared to full-length BDNF; early investigations highlighted the existence of small amounts of a 28-kDa BDNF protein that is immunoprecipitated with BDNF antibodies. This protein is generated in the endoplasmic reticulum through N-terminal cleavage of proBDNF at the Arg-Gly-Leu-Thr57-2-SerLeu site, and the cleavage is abolished when Arg54 is changed to Ala (R54A) by *in vitro* mutagenesis (Mowla et al., 2001). Truncated BDNF is less well-studied than its mature form but is thought to play a role in specific cellular contexts, potentially influencing synaptic modulation and other neural processes. The precise mechanisms and functions of BDNF and its forms are still an active area of research.

Interestingly, both proBDNF and truncated BDNF are N-glycosylated indicating that these proteins contain N-linked complex carbohydrates (Mowla et al., 2001), however, the generation of mature BDNF seems to not require the truncated form,interestingly in the U373PDX cell line, abolished generation of mature BDNF or truncated BDNF did not affect both isoforms, concluding that the truncated form is not an intermediate product of the mature form (Mowla et al., 2001).

### c. BDNF, Oxytocin and maternal care, a complicated and intriguing relationship

In the context of early-life stress (ELS), various studies have demonstrated the significant impact of maternal behavior on offspring development through the modulation of BDNF levels. In Wistar dams, maternal separation led to an enhancement in the quality of pup-directed behavior and an increase in BDNF levels in the hippocampus homogenates of the offspring, which appeared to mitigate any potential adverse effects on cognitive function. (Alves et al., 2022). In addition to that, a study on maternal and peer interactions show that individuals exposed to high levels of both maternal and peer interactions demonstrated elaborate adult agonistic competencies accompanied by high BDNF levels in the hippocampus, frontal cortex and hypothalamus (Branchi et al., 2013), thus highlighting the major role of BDNF in social interactions and more importantly in maternal behavior, that seems to have action through BDNF-TrkB signaling in oxytocin neurons (Maynard et al.,

2018), these findings were highlighted through the disruption of BDNF from promoters I and II, leading to reduced sexual receptivity and impaired maternal care in female mice, which is concomitant with decreased OT expression during development (Maynard et al., 2018). Furthermore, exposed rat pups to stressed and abusive mothers induced an increase in methylation of BDNF DNA that caused reduction in BDNF gene expression in the adult prefrontal cortex, and also altered BDNF DNA methylation in the offspring of females that had previously experienced the maltreatment regimen (Roth et al., 2009). This paper indicated that maltreatment-induced changes in methylation of BDNF DNA, and these changes were maintained through adolescence and into adulthood, even though the exposure to the abusive mothers ended at PN7 (Roth et al., 2009). The previous findings were transposed also in human, indeed, low maternal care in childhood was associated with greater DNA methylation in BDNF in blood cells at adulthood, the findings may indicate components of the epiphenotype from early life stress (Unternaehrer et al., 2015). Moreover, reduction in BDNF mRNA and protein were found in the dorsolateral prefrontal cortex of patients with schizophrenia compared to normal individuals (Weickert et al., 2003).

As it was advanced in the previous section on BDNF definition, what makes it complex is the contradictory effects that were found in the literature. Indeed in a model of gestational stress that is characterized by a reduction in maternal care, offspring from stressed dams showed higher levels of BDNF (Zuena et al., 2008). More recently in the same model, in female aged rats, PRS impacted the expression of BDNF in the ventral hippocampus and in the prefrontal cortex (Verhaeghe et al., 2021). But it's undeniable, BDNF represents a potential therapeutic avenue and it could be targeted by several investigations.

### D. Stress/anti-stress balance related diseases (focus on *postpartum* depression)

Multiple reviews have summarized the work of pioneers like Neumann, Jurek, and Windle, whose studies in both rodents and humans demonstrated a bidirectional relationship between the HPA axis and OT (Jurek and Neumann, 2018; Windle et al., 1997). OT administration has been shown to counterbalance HPA axis activation by reducing glucocorticoids (GC) release. The specifics of this inhibitory action were elucidated through pharmacological approaches in several studies. Notably, intracerebroventricular administration of OT leads to a reduction in CRH mRNA levels in the PVN in response to stress, as well as a decrease in ACTH and

corticosterone plasma levels both in the basal condition and in response to stress (Zinni et al., 2018). Furthermore, intracerebroventricular (ICV) administration of OT has been shown to decrease mouse immobility in the forced swim test, and the antidepressant effect of OT has been found to be comparable to that of imipramine which increase active behavior and reduces immobility (Jurek and Neumann, 2018). These findings are particularly relevant when considering the physiological and psychological challenges faced during the *postpartum* period. *Postpartum* depression (PPD) is a major depressive episode occurring after childbirth, affecting both the mother and her infant's well-being. The intricate neuroendocrine changes, including those involving the HPA axis and OT, play a significant role in the onset and maintenance of PPD.

Research has shown that late pregnancy OT levels could be a predictive biomarker for PPD, providing an opportunity for early diagnosis and treatment (Cevik and Alan, 2021), Furthermore a systematic review highlighted that eight studies found in women an inverse relationship between plasma OT levels and depressive symptoms (Thul et al., 2020). In line with the previous review, another systematic review highlighted hypercortisolemia is linked to transient depressive states, while hypocortisolemia is related to chronic *postpartum* depression (Seth et al., 2016). For instance, studies have highlighted that OT administration can alleviate symptoms of depression and anxiety, which are prevalent in PPD, by reducing the levels of stress hormones and enhancing mood and social bonding. Moreover, oxytocin's interaction with other neurotransmitter systems, such as the serotonergic and dopaminergic pathways, further underscores its potential as a therapeutic agent for PPD (Jurek and Neumann, 2018).

Additionally, BDNF has been identified as another critical player in PPD. As advanced in the corresponding section described above, this neurotrophin is crucial for neuroplasticity, neuronal survival, and synaptic connectivity, all of which are essential for mood regulation. Studies have shown that BDNF was higher in the pregnant group than in non-pregnant controls, and lower in the *postpartum* depression group at 6 weeks after delivery than in the perinatal non-depressed group (Y. Lee et al., 2021). In the *postpartum* depression-recovery group, the BDNF concentration increased at 6 weeks after delivery compared to that at 24 weeks of gestation (Y. Lee et al., 2021). Therapeutic strategies that increase BDNF levels, such as exercise (Huang et al., 2014), certain antidepressants (Castrén and Rantamäki, 2010),

and possibly OT administration (Bell et al., 2015; Cevik and Alan, 2021; Kimmel et al., 2016; Wang et al., 2018), have shown beneficial effects in improving depressive symptoms and cognitive function.

Finally, OT, OTR and BDNF are studied together in the context of ELS because they are crucially interconnected in regulating brain development, stress responses, and social behaviors. In fact, it was reported that OT treatment in cortical slices of 2-week-old mice led to rapid transactivation of the BDNF receptor TrkB (Mitre et al., 2022). Additionally, as it was reported above, disruption of promoters I and II of BDNF, led to reduced sexual receptivity and impaired maternal care in female mice, which is concomitant with decreased OT expression during development (Maynard et al., 2018).

*Thus, the interplay between stress/anti-stress, and BDNF offers a multifaceted approach to understanding and treating postpartum depression. The bidirectional relationship between the HPA axis and OT, as presented by Neumann, Jurek, and Windle, alongside the neuroplastic benefits of BDNF, underscores the potential for integrative therapeutic strategies in managing PPD (Jurek and Neumann, 2018; Windle et al., 1997; Zinni et al., 2018). This perspective not only enhances our understanding of PPD but also paves the way for new avenues of effective interventions. Future research should continue to explore these complexities to address a variety of mental health challenges.*

## 3. Maternal microbiota

It is now evident that the developmental trajectory can be targeted by external factors such as stress that lead to modifications in maternal care, maternal oxytocin and maternal glucocorticoids, what is more intriguing is that this developmental trajectory can also be impacted by other internal factors within the mother such as maternal microbiota. For several decades, neuroscientists primarily focused on studying the brain itself, which is understandable given its complexity and significance. However, recent research has illuminated the critical role of the gut microbiota in brain function, leading to the concept of the gut-brain axis.

The term "microbiota" refers to the community of microorganisms, including bacteria, viruses, fungi, and other microbes, that inhabit a particular environment, such as the human gut, skin, or other body parts; these environments are typically referred to as a microbiome.

These microorganisms play crucial roles in maintaining health, aiding in digestion, modulating the immune system, and protecting against pathogenic organisms (Cho and Blaser, 2012; Turnbaugh et al., 2007).

### A. Origins of microbiota

Microbiota settlement starts earlier than expected, primarily from the mother. There are two proposed modes of maternal-infant transmission: horizontal (from the external environment) and vertical (from maternal vaginal microbes during birth) (Codagnone et al., 2019; Jašarević et al., 2014). Emerging evidence suggests vertical transmission is key for initial colonization of the infant gut, influencing gastrointestinal maturation and the extraction of essential energy and macromolecules (Codagnone et al., 2019; Jašarević et al., 2014).

John Cryan and colleagues have been at the forefront of exploring how maternal microbiota influences the development and health of offspring. At birth, a newborn is exposed to the maternal vaginal microbiota, which plays a pivotal role in colonizing the infant's gut. During vaginal delivery, the proximity of the baby's head to the mother's anal region and the contact with the vaginal microbiota allows for the transfer of maternal microbes, significantly impacting the infant's microbiome (Dominguez-Bello et al., 2016). Studies have shown the mode of delivery is a major determinant of the newborn's microbiota composition. Vaginally delivered infants acquire bacterial communities similar to those found in the maternal vagina. In contrast, C-section–delivered infants are enriched in skin microbiota, indicating a different microbial colonization route (Dominguez-Bello et al., 2016). However these deficits in C-section-delivered infants were reversed by post-delivery exposition to vaginal fluids, with a profile resembling those of vaginally delivered infants, particularly so during the first week of life (Dominguez-Bello et al., 2016).

*In summary, the maternal microbiota is the first to colonize the newborn, setting the stage for the infant's future health and development. Indeed, in recent years we witnessed the rise of the gut microbiota as a major topic of research interest in biology. Studies are revealing how variations and changes in the composition of the gut microbiota influence normal physiology and contribute to diseases ranging from inflammation, psychiatric disorders to obesity.*

## B. Gut-Brain axis *vs.* HPA axis

Neuroscientists believed that the brain is the only controlling factor, for behavior, mood, endocrinology and other related functions. But soon, this vision was proven wrong. In the 1920s, Albrecht Bethe proposed ***the concept of a "second brain"*** in the gut, hinting at the complex neural network within the gastrointestinal system (Yang et al., 2020). Afterwards, Gershon's research in the 1980s highlighted the extensive network of neurons in the gut and its autonomy from the central nervous system (CNS) (Yang et al., 2020). The real question that interfered with the scientific community was and is still "how do these two brains communicate?" That led to the emergence of the **Gut-Brain Axis Concept** and research started to focus on the biochemical signaling between the gut and the brain.

The use of germ-free (GF) animals (animals raised in a completely sterile environment and free from all microorganisms), has provided one of the most significant insights. Indeed investigations in GF mice showed reduced BDNF in the cortex and hippocampus and heightened HPA axis reactivity through increased plasmatic ACTH and CORT; furthermore, those deficits were reversed through treatment using *Bifidobacterium infantis* but not with the enteropathogenic *Escherichia coli* (Sudo et al., 2004). GF mice, compared to specific pathogen-free (SPF) mice, exhibited an anxiolytic-like behavior in the EPM accompanied by neurochemical changes including impairment of serotonin receptor 1A (5HT1A) and BDNF in the central amygdala and the dentate granule layer of the hippocampus (Neufeld et al., 2011a). However, the same team showed that the colonization of GF mice with SPF feces in adulthood did not correct their anxiolytic-like behavior, underscoring the importance of a critical window and early corrective interventions (Neufeld et al., 2011b).

The HPA axis and stress is at the center of most psychiatric disorders, and studies led by Cryan have demonstrated that probiotics can reduce anxiety-like behaviors in animal models, with implications for human mental health. Cryan has explored how probiotics can influence the HPA axis, which is involved in the stress response. His findings suggest that probiotics can help modulate this axis, leading to reduced stress levels (Cryan and Dinan, 2012). Dinan and Cryan in their famous review on Biological Psychiatry the beneficial effect of probiotics on the brain, these bacteria are capable of producing and delivering neuroactive substances such as gamma-aminobutyric acid and serotonin, which act on the brain-gut axis, finally the

two pioneers presented gut bacteria (probiotic) as psychobiotic (Dinan et al., 2013). This term signifies the excitement surrounding these groundbreaking discoveries, which unveil previously unknown facets of mammalian physiology (Erdman and Poutahidis, 2016).

Taken together it's evident now that the two axes communicate with each other, but the question that intrigued neuroscientists is how do they communicate. The vagus nerve, a key part of the parasympathetic nervous system and most of the gut-brain communication are under its control, though some occur independently. Indeed, microbiota are live microorganisms, they produce metabolites such as short-chain fatty acids, which are have neuroactive properties (Cryan and Dinan, 2012), those metabolites can cross the blood brain barrier and affect neuroinflammation and neurotransmitter systems, thereby impacting mood and behavior (Bray, 2019). Some bacteria even produce neurotransmitters like GABA, serotonin, and dopamine, influencing neural signaling (Cryan and Dinan, 2012). Furthermore, some of them have a direct impact on the immune system through changes in cytokine levels. When this microbiota is disturbed it can lead to dietary competition, converting sugars into inhibitory fermentation products, produce growth substrates, generate bacteriocins, compete for gut wall binding sites, enhance gut barrier function, reduce inflammation, and stimulate immune responses (Cryan and Dinan, 2012). Beyond the periphery the gut microbiota has been shown to also play a major role in neuroinflammation. Indeed, astrocytes respond to local signals from the brain, but are also indirectly modulated by gut microbiota, studies revealed that most of the CNS diseases triggered by astrocytic dysfunction are closely associated with the dysbiosis of gut microbiome (Zhao et al., 2021).

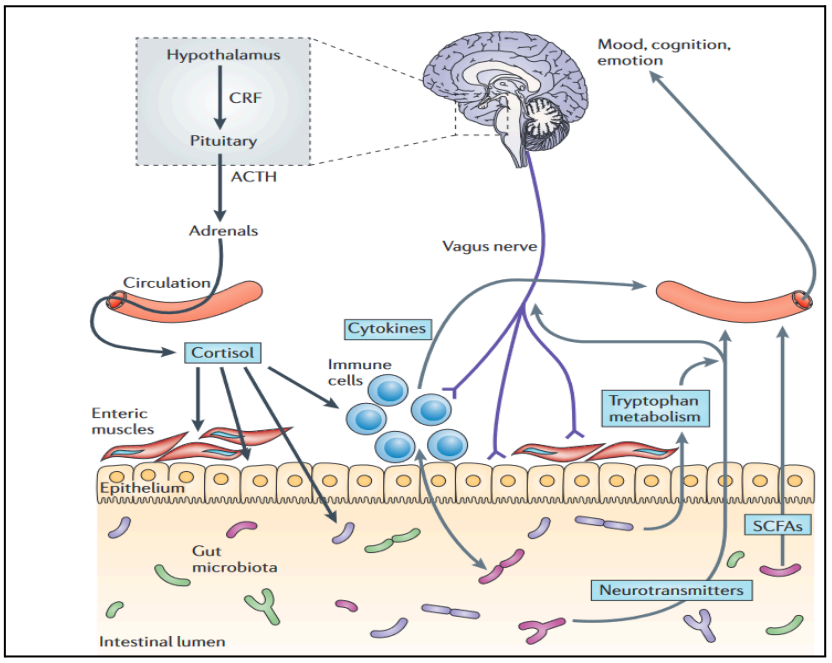


**Figure 8. Gut-brain axis, HPA axis and their communication, adapted from** (Cryan and Dinan, 2012).

The famous review 'Mind altering microorganisms' of J. Cryan and T. Dinan summarized all the potential communication pathways between the CNS and the gut (Cryan and Dinan, 2012). Some of these mechanisms and their complexity are presented in **Figure 8.**

## C. Probiotics as a new therapeutic strategy

The term "probiotic" is derived from Greek, where "pro" means "benefit" and "bios" means "life." Probiotics are beneficial bacteria that promote health and prevent diseases. They are defined as oral supplements or food products that contain a sufficient number of viable microorganisms to alter the host's microflora, offering potential health benefits (Butt et al., 2021). Nowadays, probiotics constitute a rapidly growing industry and are among the most commonly consumed food supplements worldwide. Foods such as yogurt and cheese are often supplemented with probiotics, they are also incorporated into cosmetic products and commercialized as lyophilized pills. Probiotic consumption is widely recommended by physicians, particularly gastroenterologists, who recognize their benefits for digestive health (Suez et al., 2019).

The role of probiotics in influencing health has been a topic of extensive research, with growing evidence supporting their benefits beyond gastrointestinal health, and one of the most intriguing areas of probiotic research is their potential role in modulating the brain-gut

axis and influencing neurochemical pathways, particularly those involving oxytocin, a neuropeptide critical for social bonding, stress regulation, and emotional behaviors.

The scientific interest in *Lactobacillus* strains for studying beneficial effects originated from various epidemiological studies, clinical trials, and research into the natural occurrence and health benefits of these bacteria in humans and animals. But the most evident seminal work was observations by Nobel Prize-winning scientist Élie Metchnikoff in the early 20th century. He hypothesized that the longevity of Bulgarian peasants was linked to their consumption of fermented milk products containing lactic acid bacteria, primarily *Lactobacillus* species (Metchnikoff, 1908). Nearly 100 years later, Tannock published his impactful review entitled "A Special Fondness for *Lactobacilli*" where he described that microbiologists were fans of studying *Lactobacillus* for their beneficial effect (Tannock, 2004). Later on, a lot of important investigations confirmed and highlighted its beneficial effect. Initial research on various Lactobacillus species provided promising results. In 2011, Bravo et al. (2011) demonstrated that *Lactobacillus rhamnosus* could reduce anxiety and depression-like behaviors in mice by modulating GABA receptor expression in the brain. These findings highlighted the potential of probiotics to influence mental health and behavior, encouraging scientists to explore other strains for similar or enhanced effects (Bravo et al., 2011).

The shift towards *Lactobacillus reuteri* (*L. reuteri*) began with studies that suggested specific strains might have unique properties affecting the brain-gut axis more profoundly. In 2020, *Lactobacillus reuteri* was reclassified and renamed *Limosilactobacillus reuteri* (*L. reuteri*) due to its distinct phylogenetic position (Zheng et al., 2020), highlighting its evolving and its significance in research. This has led to a race to patent new strains as potential therapeutic tools in various pathological contexts, including psychological diseases. Researchers conducted a pivotal study showing that *L. reuteri* administration in the mice dams could restore social deficits and elevated oxytocin levels in the offspring of a mouse model of maternal high-fat diet (Buffington et al., 2016), furthermore *L. reuteri* increased oxytocin levels in the PVN of the hypothalamus (Dooling et al., 2022), and *in* intestinal epithelium via secretin 1 signaling (Danhof et al., 2023). Surprinsgly, lysate of *L. reuteri* alone was sufficient to boost systemic oxytocin levels and improve wound repair capacity (Varian et al., 2017). These findings provided a mechanistic explanation for how this probiotic could influence the brain and behavior, distinguishing it from other probiotics that did not exhibit

the same specific effects such as *Bifidobacterium animalis* (Lee et al., 2017). This time in humans, and more precisely in adults with subclinical symptoms of depression, anxiety, and insomnia, 8 weeks of treatment with a mixture of *Limosilactobacillus reuteri NK33* and *Bifidobacterium adolescentis NK98*, succeeded in reducing depressive symptoms at four and eight weeks of treatment, and anxiety symptoms at four weeks, additionally an improvement in sleep quality was also reported, accompanied with a decrease in serum interleukin-6 levels (H. J. Lee et al., 2021). Furthermore, a groundbreaking study totally confirmed the major role of gut microbiota in depression and anxiety, the study named " transferring the blues", demonstrated that microbiota transplantation from depressed patients to control rats induced behavioral and physiological features characteristic of depression in animals, including anhedonia and anxiety-like behaviors, as well as alterations in tryptophan metabolism (Kelly et al., 2016). Thus highlighting *L. reuteri*'s position as a unique probiotic with specific effects on oxytocin and psychiatric disorders.

*Taken together, previous studies gave solid evidence of the major role of this probiotic in the stress/anti-stress balance, and subsequent studies followed and never failed to confirm the beneficial effect of Limosilactobacillus reuteri for influencing oxytocin levels and social behaviors, it represents a significant advancement in probiotic research. The transition from general studies on probiotics and the brain-gut axis to the specific focus of this strain stands out as a promising candidate for modulating the oxytocin system and potentially treating related neuropsychiatric conditions. However, further investigations using ELS models are needed to elucidate the underlying mechanisms and assess its effects on maternal behavior and maternal parameters, as these aspects remain underexplored in mothers.*

## III. The perinatal stress model (PRS): an epigenetic model for early-life programming of stress-related disorders

The field of developmental programming involves areas of uncertainty and ethical challenges. Apart from naturally emerging cohorts, such as those affected by the Canadian Ice Storm, or historically significant events like the Holocaust and the Dutch and Russian famines. Advancing science requires the use of animal models that can be easily tailored to specific paradigms, enabling the investigation of precise molecular mechanisms and

pathways. Numerous animal models have been developed to provide crucial insights and their contributions to the field are invaluable.

One particularly influential model is the perinatal stress (PRS) model in rats, developed by Maccari and collaborators (Maccari et al., 1995; Morley-Fletcher et al., 2003). As presented in **Figure 9**, the protocol consists in exposing pregnant dams to restraint stress under bright light from embryonic day 11 to 21, with three sessions of stress exposure per day of 45 min during the light phase. This paradigm combines prenatal stress (restraint stress and sleep disruption) with postnatal stress (increased maternal glucocorticoids and reduced maternal behavior) (Barbazanges et al., 1996; Gatta et al., 2018; Maccari et al., 1995). The offspring descending from stress dams remain with their biological mothers until weaning, continuing to experience the effects of the mother's stress and receiving inadequate maternal care. Consequently, this model includes both prenatal and postnatal stress elements, describing it more accurately as perinatal stress.

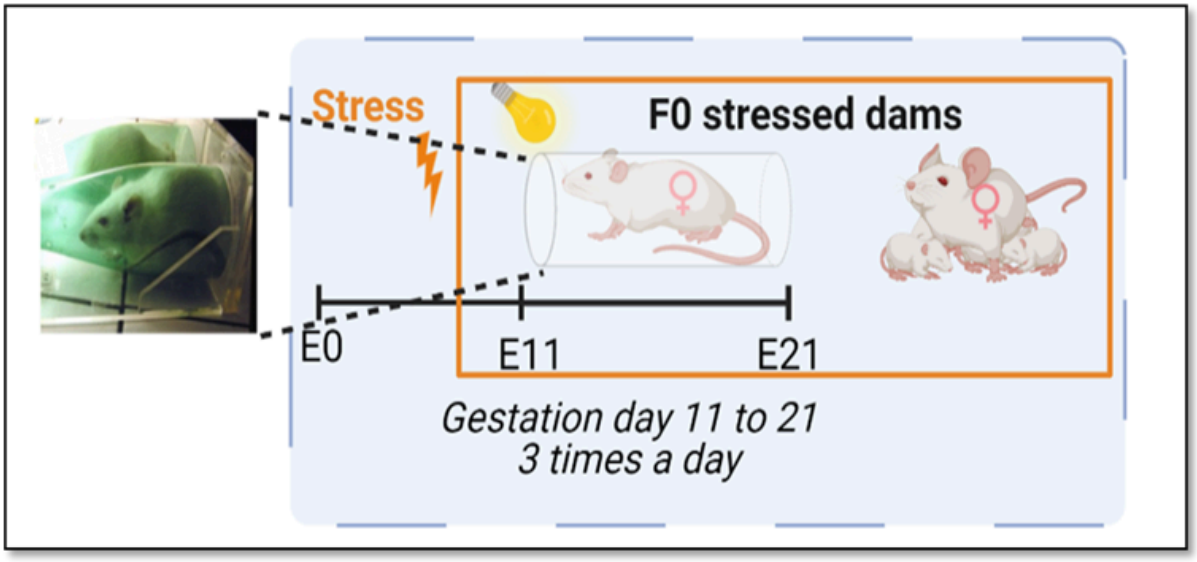


**Figure 9. Gestational stress protocol of the PeRinatal Stress (PRS) according to (Maccari et al., 1995).**

The PRS model demonstrates multiple validity parameters as an epigenetic model for stress-related disorders (Maccari et al., 2014). In the model's paradigm, the first impacted part in the mothers-pups dyad is the stressed mothers, which is translated by physiological, neurochemical, and behavioral changes. Indeed gestational stress reduced body weight gain both during pregnancy and *postpartum* periods (Darnaudéry et al., 2004), additionally, during post-weaning, stressed dams presented higher CORT levels when exposed to a 10 min novel

environment, reduced locomotor activity, as well as a reduced risk-taking behavior in the EPM, while, such differences were not evidenced in virgin females (Darnaudéry et al., 2004). Furthermore, gestational stress reduced maternal behavior toward the pups during lactation (Gatta et al., 2018; Maccari et al., 1995).

However, the stressed dams presenting deficits remain with their progeny, and the offspring until weaning continue to receive defective maternal care, incorporating both pre- and postnatal stress components (Maccari et al., 1995). In an investigation using the PRS model, cesarean sections were performed to extract fetuses and assess the impact of gestational stress. The study reported long-lasting disturbances in feeding behavior and dysfunctions associated with type 2 diabetes. This resulted in reduced body, adrenal, and pancreas weights, as well as lower plasma CORT and glucose levels in the PRS fetuses, which persisted into adulthood (Lesage et al., 2004). Furthermore, in the placenta of stressed dams, the expression of glucose transporter type-1 (GLUT1) was decreased, in association with reduced levels of glucose and growth hormone in the fetal circulation, leading in the offspring to lowered nutritional transfer, low body weight and reduced pancreatic growth at birth (Mairesse et al., 2007). As highlighted in the previous sections, there is a protective barrier between mothers and offspring mediated by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which breaks down glucocorticoids into their inactive forms (de Kloet and Meijer, 2019; Welberg et al., 2000). Remarkably, in PRS model the activity of 11β-HSD2 was strongly reduced in the placenta at embryonic day 21 (Mairesse et al., 2007), thus contributing to the increased glucocorticoid transfer to the fetus, thereby impacting its developmental trajectory. Consequently, PRS offspring exhibit a disturbed HPA axis (Maccari et al., 1995) and an imbalance in the stress/anti-stress equilibrium, though the increase of CORT levels in response to novelty stress in the PRS males (Gatta et al., 2018), surprisingly, maternal behavior correction in stressed dams using adoption or pharmacological treatment with *postpartum* carbetocin (CBT) corrected stress/anti-stress balance (Gatta et al., 2018; Maccari et al., 1995), and blocking stress-induced maternal CORT secretion by adrenalectomy in stressed dams suppressed stress consequences on the offspring, in particular the HPA axis alterations (Barbazanges et al., 1996). Moreover, an investigation precisely measured the stress hormone CORT at six different time points over 24 hours and PRS rats demonstrated a phase advance in the evening of CORT increase, suggesting that the circadian

system governing the HPA axis was vulnerable in the PRS model (Koehl et al., 1997), this vulnerability was associated with deficits in the negative feedback machinery of the HPA axis in the hippocampus, underscored by downregulation in gene expression of GR, MR, and OT in the PRS adult males, that persisted further in aged PRS males, but mitigated in offspring descending from CBT treated mothers (Gatta et al., 2018), when looking into protein expression of GR and OTR, it was also reduced in the PRS specifically in the ventral hippocampus (Morley-Fletcher et al., 2018).

Beyond the HPA axis, increasing evidence showed that PRS influenced neurotransmitters release, reducing glutamate but not GABA release in adult offspring (Morley-Fletcher et al., 2018), surprisingly neurotransmission deficits were evident at early stages (postnatal days: PND) PND14 and PND22, where PRS pups showed a reduced expression of the gamma 2 subunits of GABA A receptors in the amygdala (Laloux et al., 2012). Furthermore, the impact of PRS on ***glutamate transmission*** was highlighted selectively in the ventral hippocampus, with an impairment of depolarization-evoked glutamate release in synaptosomes and reduced expression of synaptic vesicle-associated proteins (Marrocco et al., 2012). These changes in metabotropic glutamate receptors (mGluRs) are detectable very early at PND10, including reduced expression of mGluR1 and mGluR5 receptors in PRS rats, with a decline in mGluR2/3 receptor expression occurring only after weaning (Laloux et al., 2012). These glutamatergic changes persisted into adulthood and were observed in aged PRS animals, showing a notable decrease in mGluR1 and mGluR5 receptors in the hippocampus specifically in males, and a reduction in mGluR2/3 in both males and females (Laloux et al., 2012; Van Waes et al., 2011; Zuena et al., 2008). In the hippocampus, specifically the ventral region, PRS males presented lower levels of OTR, GR, mGluR2/3, and mGluR5 (Morley-Fletcher et al., 2018), however, chronic treatment with S 47445 reversed OTR and GR levels, but not mGluR2/3 (Morley-Fletcher et al., 2018). Moreover hippocampal neurogenesis and neuronal plasticity were reduced by PRS in association with reduced PSA-NCAM (PolySiAlylated Neural Cell Adhesion Molecule) in the dentate gyrus of ventral and total hippocampus but not in the dorsal (Morley-Fletcher et al., 2011). Moreover, BDNF supports the growth of new neurons and is crucial for the long-term survival of newborn neurons in the hippocampus. Interestingly, levels of both BDNF and proBDNF increased in

the ventral hippocampus of PRS males but not females, with no change observed in the dorsal hippocampus in either sex (Zuena et al., 2008).

Rescently, a study highlighted through plasma metabolomic analysis significant changes linked to PRS in aged male rats, affecting biomarkers of secondary bile acid metabolism in plasma and glutathione metabolism in the frontal cortex (Morley-Fletcher et al., 2024). *Postpartum* CBT was effective in reversing these metabolite alterations, indicating its potential disease-specific benefits. The metabolomic signatures of PRS were associated with sustained cognitive and emotional changes, as well as endocrinological disturbances (Morley-Fletcher et al., 2024).

As expected, the aforementioned deficits were associated with a deficient behavioral phenotype which manifested early during development. Indeed, offspring of stressed dams at PND14 exhibited increased ultrasonic vocalizations (USV) in response to isolation from their mothers, and a delayed suppression of USV production when exposed to an unfamiliar male odor (Laloux et al., 2012). Similarly, in adulthood, PRS offspring demonstrated increased immobility in the forced swim test (Morley-Fletcher et al., 2003), reduced risk-taking behavior as evidenced by decreased time spent in the open arms of the EPM, and an increased latency to enter the open arms. A similar trend was observed in the Light/Dark test (Morley-Fletcher et al., 2003). Chronic treatment with antidepressants or the ampakine S 47445 successfully ameliorated these behavioral deficits, and biochemical alterations highlighting the predictive validity of this preclinical model for studying stress-related disorders (Morley-Fletcher et al., 2018, 2011).

Overall, the PRS rats model serves as a well-established model for studying the long-term effects of early life stress on behavior and neurobiology, unfortunately ELS studies as most scientific studies focus on males, reducing considerably our knowledge for females-related deficit and sex differences in stress response. Despite long-standing hypotheses regarding sexual dimorphism in PRS effects, few studies have explicitly examined this issue. In adult male and female rats, PRS has been shown to generate two distinct behavioral and neurochemical profiles, males presented reduced risk-taking behavior in the EPM, impaired neurogenesis, neuroplasticity (BDNF and proBDNF) and neurotransmission related-proteins (mGluR1/5) in the ventral hippocampus (Zuena et al., 2008). Conversely, female PRS rats

exhibit increased risk-taking behavior and reduced mGluR2/3, but no significant changes in hippocampal neurogenesis, BDNF levels and mGluR1/5 (Zuena et al., 2008). A more recent study further supports sex-specific responses to PRS that persisted in aged rats and reported that females were more protected than males from stress (Verhaeghe et al., 2021), identifying PRS-related demasculinization in the glutamatergic transmission, programming brain aging differently in males and females, highlighting sex-specific responses to early stress and suggesting females may be more resilient to age-related problems (Verhaeghe et al., 2021).

*Taken together, the PRS model in both mothers and offspring demonstrates a wide range of deficits, making it a valid preclinical model for various disorders. These include psychiatric and depressive disorders, inflammatory and metabolic disorders, memory and motor deficits, and maternal-deficit related disorders. The mechanisms underlying these perturbations are still under investigation, and the persistence of these deficits remains uncertain. However, previous reports on PRS suggest that ELS effects can persist into adulthood and even into old age in animals. The current challenge is to determine how long these effects last over the generations; which is never proven in this model and whether they are reversible, considering that epigenetic changes have the potential to be reversed.*

## Aims of the thesis

As addressed in the introduction, during critical periods of development such as prenatal and postnatal life, various environmental factors including stress can profoundly impact the developmental trajectory of diseases, influencing behaviors and molecular pathways leaving lasting imprints on the brain. These molecular imprints can serve as biomarkers, facilitating translation to human applications.

Furthermore, evidence supports the inheritance of stress-related deficits through the capacity of maternal behavior to reprogram offspring. However, there is a notable lack of research into strategies for mitigating these stress-related deficits, particularly in reversing early life programming. Significant gaps remain, especially regarding early-life interventions during critical periods such as the *postpartum* period.

Within this context, the main aim of my thesis is to study maternal-mediated mechanisms in the epigenetic programming induced by maternal stress through oxytocinergic activation in mitigating stress-related deficits. In general, we investigated whether the programming related to the PRS phenotype was mediated by maternal behavior and its associated endocrine environment, particularly to the stress/anti-stress balance parameters. First, to firmly establish that the mechanisms mediated by the mother are epigenetic. Second, we performed corrective interventions on maternal behavior by activating the oxytocinergic system during the *postpartum* period. We traced stress-related deficits across generations, using an analog of OT named carbetocin, or with an alternative non-pharmacological approach via probiotic supplementations (*Limosilactobacillus reuteri*). This involves examining whether PRS deficits persist via deficiencies in maternal behavior and whether correcting this behavior through oxytocinergic activation during the *postpartum* period in F0 mothers reverses these deficits and, importantly, whether this correction persists across multiple generations in an inter- or transgenerational manner. This thesis is divided into two chapters each one subdivided into two parts:

**Chapter One: Epigenetic inheritance of the PRS:** Aims to investigate the persistence of PRS-related deficits through the maternal line, in both intergenerational and transgenerational transmission manner, and highlight whether oxytocinergic activation through pharmacologic

approach in F0 dams during *postpartum* weeks was strong enough to correct PRS deficits across generations. The paradigm used in this part is summarized in **Figure 10.**

**A)** The first part of this chapter aimed to investigate the intergenerational transmission of stress (from F0 to F2 generation) and highlight the role of maternal behavior in correcting stress deficits using *postpartum* oxytocinergic activation *via* carbetocin (CBT) treatments. The investigation includes both behavioral, hormonal and molecular analyses, with an extensive characterization of the mothers and their offspring as well as the mothers-offspring dyad, by focusing on the HPA axis and the stress anti-stress balance. The goal was to determine whether CBT can mitigate stress effects in offspring through enhanced maternal behavior across generations.

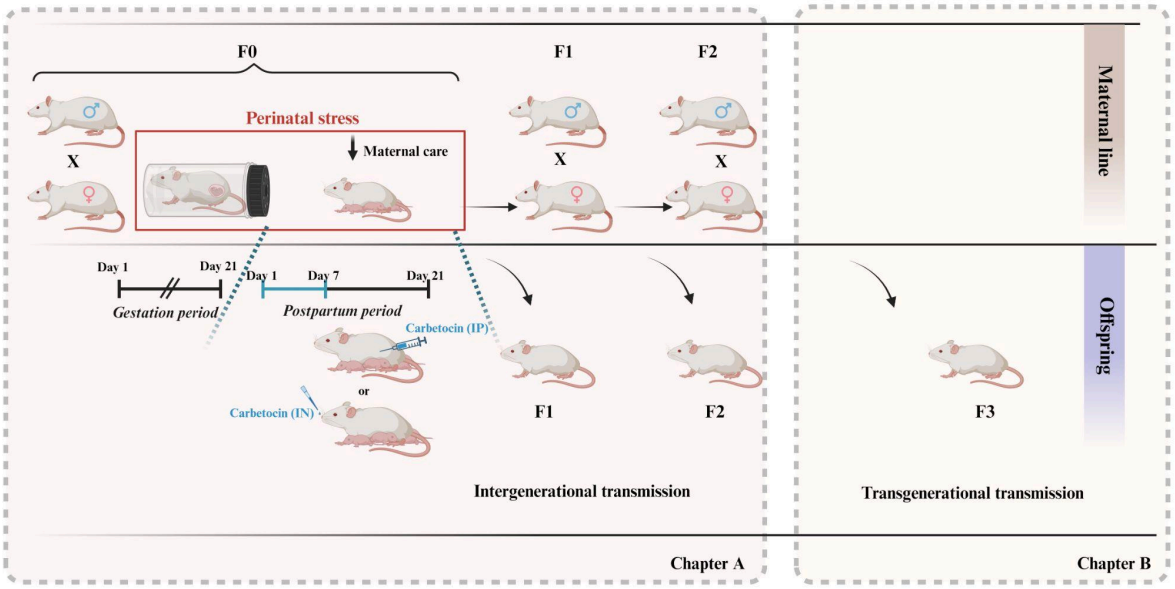


**Figure 10. Perinatal stress transmission and the setup of generations from F0 to F3 using the maternal line (*Chapter 1*). Females from the studied groups (control, stressed/PRS, and stressed/PRS+ treated) were mated with control non-stressed males to generate different generations. In the present figure, only the stressed/PRS group is presented.**

**B)** In the second part we explored the transgenerational transmission (from F0 to F3 generation) of epigenetic and molecular mechanisms in maternal-mediated programming of PRS. Here we addressed the persistence of gestational stress occurring in F0 mothers and its programming effects on behavioral and molecular changes up to the F3 PRS offspring, representing an inheritance from great-grandmothers to offspring, and the efficacy of

*postpartum* intraperitoneal (IP) as well as intranasal (IN) administration of CBT in F0 dams in rescuing stress effects in both mothers and offspring (F3).

***Chapter Two***: **The anti-stress effect of *Limosilactobacillus reuteri* in mothers-offspring dyad:** aims to address the anti-stress (oxytocin) effect of *L. reuteri* in the PRS model. The used paradigm to address the question is represented in **Figure 11** that is also divided in two parts.

**A)** The first part of chapter two focuses on rat mothers, investigating whether supplementation with the probiotic *L. reuteri* during the first *postpartum* week can correct maternal behavior through oxytocinergic activation (**Figure 11**). We addressed whether this bacterium could enhance maternal behavior through behavioral analysis and whether it could increase peripheral OT levels and reduce stress effects as indicated by CORT levels. Additionally, we conducted molecular investigations to elucidate the possible mechanisms underlying the probiotic's effects, targeting the hypothalamus—the central structure of OT production. We also aimed to determine whether BDNF, a specific biomarker of neuroplasticity, is involved in the effects of *L. reuteri* on maternal stress.

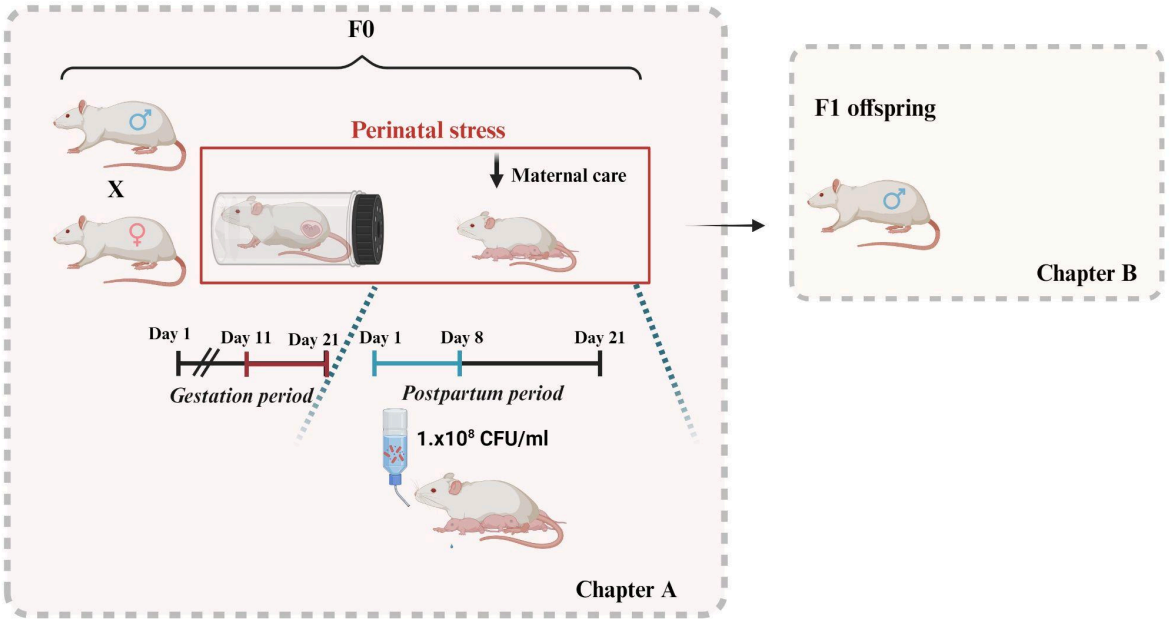


**Figure 11. Overview of the General Paradigm of perinatal Stress and *Postpartum* intervention with *Limosilactobacillus reuteri* used in Chapter 2.**

**B) The second part of chapter two of this PhD thesis,** by following the exploration of the potential corrective effects of *L. reuteri* on maternal behavior, investigated whether this correction could effectively rescue behavioral and hormonal deficits in male PRS offspring. We placed particular emphasis on the stress hormone corticosterone and a hallmark behavior in the stress response, specifically "risk-taking behavior," in the EPM. Additionally, recognizing that neuroplasticity is fundamental to brain programming, we also investigated the potential mechanisms that implicate BDNF protein expression for its brain plasticity capacity as well as OTR for its oxytocinergic activity. The PRS model ,as identified in previous studies, has been associated with reduced neuroplasticity through decreased levels of PSA-NCAM (polysialylated neural cell adhesion molecule) (Morley-Fletcher et al., 2011) and presents a proinflammatory profile characterized by increased systemic cytokines (Vanbesien-Mailliot et al., 2007). For that reason we started exploring enzymes involved in polysialylation and inflammation, namely ST8SIA4 and ST6GAL1. This chapter provides an ongoing perspective on the potential reprogramming effect of probiotics, assessing whether the findings align with those observed when a direct agonist of OTR was used, as demonstrated in previous reports.

*This PhD thesis seeks to deepen the understanding of the long-term effects of early life stress and its transmission across generations, highlighting maternal behavior as a crucial mediator in this process. Significantly, the research points to promising therapeutic interventions for the postpartum period, such as probiotics or carbetocin, which show potential in reversing stress-induced programming. This underscores the epigenetic dimensions of early life stress.*

# Results

# Chapter One: Epigenetic inheritance of the perinatal stress

## A. Intergenerational transmission of gestational stress *(Article n° 1 in preparation)*

Early-life experiments can impact the developmental trajectory of health and disease, more importantly adverse experiments such as stress during critical period like gestation can have a permanent molecular and behavioral blueprint that can persist until adulthood (Marrocco et al., 2012; Zuena et al., 2008) and even at aging (Gatta et al., 2018; Verhaeghe et al., 2021) suggesting the involvement of the epigenetic mechanism on this long-term programming. At this purpose, pioneering work highlighted that maternal behavior can have epigenetic programming capacities on the offspring (Roth et al., 2009; Weaver et al., 2004). This epigenetic programing gives the hope for reversal of these deficits because epigenetic alterations are not irreversible like a genetic mutation. Indeed stress can programs the offspring through multiple generations (Boscardin et al., 2022), and the correction of maternal behavior through adoption (Maccari et al., 1995) and pharmacological interventions using carbetocin (CBT) (Gatta et al., 2018) mitigate stress-related deficits in the offspring.

In this chapter, using the perinatal stress model (PRS) we wanted to investigate if stress programming can impact beyond the exposed F0 dams and the F1 offspring; so, we wanted to highlight the intergenerational transmission of gestational stress from F0 to F2 generation through the maternal line, and prove the pivotal role of maternal behavior through pharmacological activation using CBT solely during the 1st week of the *postpartum* period and only in F0 dams. First, we characterized PRS-related deficits both in the mothers (F0-F1) and their offspring (F1-F2) by assessing behavioral and molecular parameters. Secondly, we moved forward to highlight the role maternal behavior in correcting the stress deficits in the offspring by modulating maternal care using *postpartum* CBT, then we looked to the stress/anti-stress balance, in both the plasma and the hippocampus, a key structure in response to stress, and we also concorded our molecular investigation to behavioral parameters such as maternal behavior, risk-taking behavior in the EPM.

Finally, the findings of this chapter, presents hope for breaking the persistence and transmission of traumatic events occurring during vulnerable periods of life.

## Article n° 1 in preparation

# Transient *postpartum* activation of oxytocin receptor prevents postnatal intergenerational inheritance of early life stress

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## ABSTRACT

The regulation of maternal care by glucocorticoids and oxytocin is crucial in shaping the behavioral and physiological functions associated with the proper development of offspring and its long-term effects, which can span across generations. This study explored the stress/anti-stress interplay in the transmission of perinatal stress and assessed the potential of the oxytocinergic agonist carbetocin to prevent intergenerational stress inheritance. We used two generations of rats, F1 and F2, both male and female, perinatally stressed (PRS), and their mothers (F0 and F1). The first-generation PRS female rats (F1) were mated with naïve males to create an intergenerational PRS model along the maternal line. Our findings revealed intergenerational transmission of maternal stress, characterized by a like-mother-like-daughter alteration of maternal behavior and an impaired stress/anti-stress balance transmitted across generations. Remarkably, transient activation via carbetocin of the positive feedback loop between oxytocin and maternal care in stressed F0 rat mothers during the first postpartum week was transmitted to F1 PRS mothers. This intervention reversed the downregulation of stress-response related genes and their behavioral correlates up to the F2 PRS offspring of both sexes. Our results suggest that maternal oxytocin activation during the critical window in the first week of lactation in F0 mothers represents an optimal therapeutic strategy to prevent the vicious cycle of intergenerational early-life stress inheritance. By restoring the stress/anti-stress balance, this approach enhances the individual's ability to respond to stress in adulthood as well as of the future generation.

## INTRODUCTION

Glucocorticoids and oxytocin (OT) play essential roles in modulating the body's stress and anti-stress responses. These hormones are pivotal in regulating maternal behaviors, significantly influencing the behavioral and physiological development of the offspring. Understanding the interplay between glucocorticoids and oxytocin in the epigenetic transmission of maternal programming, provides critical insights into the mechanisms underlying stress resilience and the transmission of behavioral traits across generations.

Indeed, early life stress (ELS) involves epigenetic mechanisms transmitted via changes in maternal care from mother to daughter (Champagne et al., 2001; Champagne and Meaney, 2006; Francis et al., 1999), modifying stress responses and impacting behavioral and cognitive functions in adulthood in both animal models (Maccari et al., 2014, 1995; Roth et al., 2009; Weaver et al., 2004) and humans (Eriksson et al., 2014; Turecki and Meaney, 2016; Yehuda et al., 2008). Exposure to stress (during perinatal periods) disrupts maternal care, and increased maternal corticosterone passed to pups via milk, shapes the offspring phenotype by disturbing the hypothalamic-pituitary-adrenal (HPA) axis (Angelucci et al., 1985; Barbazanges et al., 1996a; Maccari et al., 1995). Like glucocorticoids, OT can be epigenetically tuned by early life events. Hence, perturbed OT transmission is linked to poor maternal behavior (Sanson and Bosch, 2022), and inadequate mothering is associated with defective OT signaling, reduced OT gene expression, and increased OT promoter methylation during pregnancy (Toepfer et al., 2019).

Perinatal stress (PRS) in rats induces long-term behavioral changes and hippocampal molecular alterations, predicted by reduced maternal behavior due to gestational stress. Truly, maternal behavior is at the core of the PRS lifelong programming (Maccari et al., 1995) and several maternal-mediated mechanisms have been identified, including alterations in glucocorticoids, glutamate, and glucose (Maccari et al., 2017). PRS males display reduced hippocampal glutamatergic transmission and low risk-taking behavior, with both sexes showing lifelong disruption in HPA axis activity, insulin resistance, disrupted circadian rhythms, and reduced mGlu2/3 receptors. Behavioral alterations, HPA axis impairment, and sex differences in PRS impact are persistent and observed up to aging (Marrocco et al., 2020; Verhaeghe et al., 2021).

Behavioral and environmental early interventions targeting the mother and/or the stress/anti-stress balance, such as environmental enrichment in rodents (van Praag et al., 2000) or parenting support programs (Webster-Stratton and Reid, 2010), have been proven effective in reversing the impact of maladaptive programming induced by maternal stress. In particular, enhancing maternal care (and therefore the OT system in the mother) through early adoption and foster care can reverse the effects of maternal stress on impaired corticosterone feedback in adult rat offspring (Maccari et al., 1995). Additionally, postpartum carbetocin, an OT analog, restores maternal care in stressed dams and corrects the stress response and associated behaviors in the stressed offspring (Gatta et al., 2018; Morley-Fletcher et al., 2024). However, the efficacy of these strategies has focused to the prevention of the

long-term effect of ELS on the immediate offspring generation, despite increasing evidence of ELS transmission across generations (Boscardin et al., 2022; Klengel et al., 2016, 2016).

Here, we explored the stress/anti-stress interplay in the intergenerational transmission of maternal stress and PRS long-term programming and assessed the potential of carbetocin to prevent intergenerational stress inheritance. We used two generations of PRS rats (F1 and F2), both males and females, and their mothers (F0 and F1). First-generation (F1) PRS female rats were mated with naïve males to create a maternal line intergenerational PRS model. From the F0 stressed mother treated or not with postpartum carbetocin, up to the F2 PRS generation, we identified in the molecular and behavioral correlates, the key epigenetic candidates in the intergenerational transmission of maternal programming.

## METHODS

### Ethics

All experiments followed the rules of Directive 2019/10/10 of the Council of the European Communities and the Comité d'Ethique CEEA-75 (Comité d'Ethique en Expérimentation Animale Nord-Pas de Calais). This project was approved by the MESRI (Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation ; authorization #33654).

**Animals** Nulliparous Sprague Dawley female rats (Charles River, France) weighing approximately 250 g, were purchased from Charles River (France) and housed under standard conditions with a 12 h light/dark cycle (lights on 7 am: lights off 7 pm). Upon arrival in the animal facility, the animals were placed in large cages (between 3 to 5 animals per cage) for 10 days to acclimatize and synchronize their estrous cycle. After group housing (five females/cage) for two weeks, each female was individually housed for one week with a sexually experienced male rat. Following that, a gain of at least 10 grams was considered as an index of pregnant status. Pregnant females were then randomly assigned to either the stress or the control group.

**Maternal line and experimental design** F0 females from the control or stressed groups were mated with control naive males to generate the F1 offspring, then females F1 from control, stressed or stressed + Cbt F0 mothers, were mated with control naive males to generate the F2 offspring. A separate set of mothers was used for the analysis of effect of carbetocine treatment across generations. As for the offspring, two separate sets different were considered: Set-1 included PRS and control unstressed F1 and F2 male and female rat offspring, while Set-2 included control unstressed, PRS + Veh or PRS + Cbt rat offspring, i.e. the offspring from dams treated with either saline or carbetocin during the postpartum period. Overall, 3 different groups combinations were considered along the study; Control (CONT); Stress/PRS + Veh or Stress/PRS + Cbt, and the corresponding control unstressed and untreated groups (CONT), including F0-F1 mothers and their respective offspring F1-F2.

**Maternal stress procedure.** Gestational stress was performed on the F0 generation according to our standard protocol (Maccari et al., 1995; Morley-Fletcher et al., 2018). Starting from the 11th day of pregnancy until delivery, the dams experienced three daily stress sessions during the light phase, each lasting 45 minutes (from 8 am to 5 pm), during which they were placed in transparent plexiglass cylinders with conical end caps and exposed to bright light (979 Lux). Control unstressed females were left undisturbed during gestation in their home cages but were handled twice a week during bodyweight gain evaluation. After birth, the pups remained undisturbed with their mother until weaning, which took place on pnd 21.

**Analyses in the mother during gestation and postpartum period**

***Spontaneous abortions evaluation*** During gestation, the weight was supervised in all rats every three days. A drastic loss of weight >10g was considered as spontaneous abortion. Following this, a ratio was calculated between the number of abortions in each experimental group in comparison to the number of abortions observed (relative frequency).

***Nest quality*** Observations of nest architecture in the dams were conducted each morning before 10 am during the final two days of gestation. The scoring system ranged from 4 to 0 (Rosenblatt, 1975). A score of 4 (maximum) indicated that the nest was fully prepared for labor and cleared of all sawdust. Conversely, absence of nest received a score of 0.

***Reactivity to novelty on pp1 (novel object test)*** The test was conducted for 5 minutes by introducing a rectangular-shaped wooden object into the home cage of lactating dams (pp1 was defined as the day after birth). During this time, the dam's behaviors, including time spent exploring the object, self-grooming, and contact with pups, were recorded and presented as % of time in spent behavior.

***Maternal care (pp1-pp7)*** The active behavior of the mother in the nest (nursing behavior, licking, carrying pups, and arched back over pups) was scored every minute (60 observations/h with 1 h of observation per day, from 7 am to 8 am) with small infrared cameras (AMC, France) on the animal cage rack where cages containing lactating females were placed. The data obtained were expressed as a percentage of licking/nursing with respect to the total number of observations, thus allowing for a comprehensive understanding of maternal response to the gestational stress as previously reported (Gatta et al. 2018; Morley-Fletcher et al. 2024). The rhythm of maternal behavior was also monitored at pp3 (time-points: 15h, 19h, 22h, 2h, 7h) to detect any circadian alteration.

***Maternal response to pups' separation test (pp7)*** Maternal responsivity following a 15-minute separation from pups was investigated. After reuniting, the latency to initiate contact with the pups was measured.

***Maternal aggressive behavior (pp7)*** A different group of dams was used to evaluate maternal aggressive behavior, a male was introduced into the home cage of lactating female rats to assess their aggressive behavior, with pups protected by a glass lid. Over a 10-minute period, offensive contacts (biting, chasing, attacking) towards the intruder were recorded, then presented in % of aggressive contacts.

***Maternal hoarding behavior (pp15)*** Lactating females and their home cage with the pups were placed in an open field. Conventional chow pellets were placed 0.35 meters in front of the cage door. A bright light was lit to increase motivation to hoard. The session lasted 30 minutes, starting when the cage door was opened, allowing the dam to carry food back to its home. The number of hoarded pellets in the home cage was measured and presented in %.

***Maternal stress/anti-stress balance measurements***: We assessed basal plasma levels of OT and CORT by ELISA (OT: sensitivity 9.4 pg/mL, CUSABIO #CSB-E14197r; CORT: sensitivity 6.1 ng/mL, Demeditec #DEV9922), and hormonal levels were calculated using a calibration curve. Tail blood (E20) or trunk blood (PP26) from F0 and F1 dams were collected using EDTA and protease inhibitors. The blood was centrifuged for 15 minutes at 1000 g at 4°C to isolate plasma, which was then stored at -20°C until analysis.

***Postpartum carbetocin treatments in F0 mothers*** Carbetocin (Cbt, SP080756, Polypeptide group, Strasbourg, France) a specific agonist of oxytocin receptors (OTR) was administered intraperitoneally to lactating dams at a dose of 1 mg/kg from postpartum day 1 to 7. The vehicle (saline) was given to the control unstressed dams and a group of stressed females. The dose and route of administration were based on previous studies (Gatta et al., 2018; Morley-Fletcher et al., 2024).

**Analyses in the offspring**

PRS and control unstressed male and female rats of F1 and F2 generation were assessed when adult at 3-5 months of age.

***Risk-taking behavior in the Elevated-Plus Maze (EPM)*** We used EPM according to our previous protocol (Marrocco et al., 2012). Briefly, the rat was placed in the maze center, initially facing a closed arm. Video tracking with EthoVision software (Noldus, The Netherlands) recorded visits and time spent in each arm for a duration of 5 minutes. Risk-taking behavior was represented by the % time spent in the open arms, and entries to open arms and entries to closed arms.

***Stress/anti-stress balance measurements after novelty stress exposure*** Animals were subjected to the novelty stress after being placed in a transparent cylindrical Plexiglas cage (30 cm diameter, 50 cm high) with a bit of sawdust and a bright light (400 lx). Plasma was extracted from four blood samples collected from the tail vein before stress (T0) and at 30, 75, and 120 minutes afterward. The blood was collected using EDTA and protease inhibitors, then centrifuged for 15 minutes at 1000 g at 4°C to

isolate the plasma, which was stored at -20°C until analysis. Plasma levels of oxytocin (OT) and corticosterone (CORT) were assessed using ELISA (OT: sensitivity 9.4 pg/mL, CUSABIO #CSB-E14197r; CORT: sensitivity 6.1 ng/mL, Demeditec #DEV9922), and data were expressed as area under the curve or delta to basal (T0) level.

***Measurement of proteins expression by Western blotting*** We used whole hippocampus to measure protein expression in total homogenates according to a previous established protocol (Morley-Fletcher et al., 2018) with antibodies against BDNF (1:500, #282051-AP, Proteintech), and mGlu2/3 receptors (1:1000; catalog #06-676; Millipore). Data were normalized to the expression of β-actin (1:1000, #A5316, Sigma) and then expressed as a ratio of the CONT rats' group.

**Stress/anti-stress balance mRNA analysis in the hippocampus** We used two different sets of adult offspring for gene expression analysis: Set-1 included PRS and control unstressed F1 and F2 male rats while Set-2 included control unstressed, PRS + Veh or PRS + Cbt rats (i.e. the offspring from dams treated with either saline or carbetocin during the postpartum period) and both males and females. RNA was extracted from the hippocampus using TRI reagent (T9424). RNA concentration was determined using a Nanodrop (ND1000, Labtech, Germany), and quality was verified by RIN (RNA Integrity Number; Bioanalyzer 2100, Agilent Technologies, France). Retrotranscription was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, France). Transcript levels were measured by real-time PCR using either SYBR Green qPCR (for set-1 animals) or TaqMan assays (for set-2 animals) (Applied Biosystems, France). The following primers were used for SYBR Green qPCR: glucocorticoid receptor (GR, Nr3c1): forward ccatcgtcaaaagggaaggg; reverse cagctaacatctctgggaat; mineralocorticoid receptor (MR, Nr3c2): forward gattccaggtcgtgaagtggg; reverse agaggagttggctgttcgtg; brain-derived neurotrophic factor (BDNF): forward caggttcgagaggtctgacga; reverse cgcgtccttatggttttcttcg; hypoxanthine-guanine phosphoribosyltransferase (HPRT): forward cgaagccacactgctgaaca; reverse accctctaggaagcgagtgt. The following TaqMan real-time PCR probes were used: glucocorticoid receptor (GR, Nr3c1, Rn00561369_m1); mineralocorticoid receptor (MR, Nr3c2, Rn00565562_m1); oxytocin/neurophysin 1 prepropeptide (OT, Rn00564446_g1. Transcript levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn001775763_g1) expression. Data acquisition (threshold cycle, Ct) was performed, and the amount of DNA was normalized to the quantity of the housekeeping gene HPRT for SYBR Green qPCR or GAPDH for TaqMan assays. Finally, the relative expression of mRNA was expressed as a relative to CONT of each sex-generation-matched group.

***Global methylation in the hippocampus by Luminometric Methylation Assay*** We measured global genomic DNA methylation in hippocampal tissue of F1 PRS and CONT unstressed males (pnd 25) by using LUminometric Methylation Assay (LUMA) following an adapted protocol (Karimi et al., 2006). DNA extraction and quantification was performed using DNeasy Tissue Kit (Qiagen cat# 69504). Genomic DNA (1 ug) was cleaved with the CpG methylation sensitive restriction enzyme HpaII and its

CpG methylation insensitive isoschizomer MspI in two parallel reactions, with EcoRI included in all reactions as a normalization reference (HpaII + EcoRI or MspI + EcoRI). Following DNA digestion, samples were placed in a Pyrosequencer and peak heights as well as the HpaII/MspI ratio (index of DNA methylation) were calculated using the PSQ96 software. If DNA is completely unmethylated, the HpaII/MspI ratio would be 1.0, and if DNA is 100% methylated the HpaII/MspI ratio would approach zero. Data were expressed in percentage of methylation (1- HpaII/MspI x100).

**Targeted DNA methylation of GR, MR and BDNF gene promoter** DNA isolated from hippocampus of F1 and F2 PRS and CONT male rats was bisulfite converted and methylation status of CpG dinucleotides within the promoter region of rat GR type II, MR-TYPE I and brain-derived neurotrophic factor (BDNF) were analyzed (McGill et al., 2006) by pyrosequencing. Bisulfite modification was carried out using Zymo Research EZ Methylation kit (Cat. #D5002 or D5004). 200 - 500 ng of sample DNA was used for bisulfite modification followed by the PCR amplification. Bisulfite modification and pyrosequencing were performed by EpigenDx (Hopkinton, MA, USA).

**Statistical analyses** Statistical analyses were conducted using STATISTICA 8.0 (StatSoft Inc.). Normality of data distribution was assessed using the Shapiro-Wilk test. Depending on the outcome, either a one-way ANOVA with Newman-Keuls post-hoc tests for parametric data, or the Mann-Whitney U test for non-parametric data was applied. Independent variables included group (stress or PRS *vs.* CONT) and treatment (Cbt *vs.* PRS or CONT). A p-value of $< 0.05$ was considered as statistically significant. The Pearson test was used for correlation analysis within and between generations. Graphical representations were created using GraphPad Prism version 10.2.3.

## RESULTS

### Gestational stress reduced mother's behavior and stress-antistress balance in F0 females and modulated stress transmission to F1 PRS offspring

We thoroughly examined the physiological and behavioral profile of the F0-stressed mother throughout gestation and postpartum (pp) periods, as illustrated in **Figure 1A**.

Throughout the entire journey of motherhood, from gestation to postpartum, gestational stress exerted a profound influence on the mother. Along gestation (E20), stressed mothers (F0) displayed an impaired stress/anti-stress balance (**Figure 1B**), with increased CORT levels and reduced OT levels (*stress effect*, CORT, n=6-9 dams/F0 group, $F_{(1,13)}=6.78$, p=0.02; OT n=5 dams/F0 group, $F_{(1,8)}=8.33$, p=0.02) as well as a reduced risk-taking behavior (**Figure 1C**) in the light/dark test (reduced time spent in the light compartment) (n=6-8 dams/F0 group, *stress effect*, $F_{(1,12)}=38.42$, p= 0.00004). Gestational stress also impacted the quality of nest by significantly reducing nest building score (**Figure 1D)** during the

peripartum period (i.e. the two days before and the day of parturition) thus indicating a delayed onset of maternal behavior (n=16-20 dams/F0 group, *stress effect*, $F_{(1,34)}$=19.94, p=0.00008). During lactation, stressed mothers also presented an avoidance profile to the novel object in the cage at pp1 day **(Figure 1E)**, with reduced time spent exploring the object and greater time dedicated to self-grooming (n=8-9 dams/F0 group, *stress effect*, % time exploring object, $F_{(1,15)}$= 27.68, p=0.0001; % time self-grooming $F_{(1,15)}$=5,08, p=0.03), while time spent with the pups was reduced but with no differences between stressed and control mothers (*stress effect* n.s. $F_{(1,15)}$=0.08, p=0.77). A major impact of gestational stress was observed on the mother-infant dyad as stressed mothers displayed reduced maternal care during the 1st week of lactation (**Figure 1F**; *stress effect*, n=6-10 dams/F0 group, pp1-pp7 graph, $F_{(1,14)}$=43.85, p=0.00001). Moreover on pp3, gestational stress also flattened the rhythm of nursing behavior during the day and night cycle (rhythm at pp3 graph; n=6-10, *stress effect*, $F_{(1,14)}$=4.87, p=0.04). Indeed, while CONT F0 dams presented fluctuation in maternal care with higher levels at 15h and 7h *vs.* 19h (light /dark shift), this was not observed in the stressed dams. During the second week of lactation, we also assessed aggressive behavior and hoarding behavior on postpartum day (pp) 7 and pp 15, respectively (**Figure 1G**). Both behaviors were impaired in stressed mothers. Specifically, there was a reduction in the number of offensive contacts towards a male intruder and a lower number of pellets collected outside the home cage in stressed mothers (*stress effect*, maternal aggressive behavior, n= 5-8 dams/F0 group, $F_{(1,11)}$ =5.57, p= 0.03; maternal hoarding behavior, n=5-7 dams/F0 group, $F_{(1,10)}$=8.49, p=0.015).

We then analyzed the F1 adult descendants of stressed mothers, named PRS offspring in comparison to the control unstressed offspring (F1 protocol, **Figure 1H**). PRS phenotype in male offspring was programmed by maternal stress through the altered stress/anti-stress balance. Indeed, as for the F0 stressed mother, F1 PRS males displayed higher CORT and lower OT plasma levels after exposure to a 30-min novelty stress (**Figure 1I**; *PRS effect*, CORT n=7 rats/F1 group, $F_{(1,12)}$=8.62 p=0.012; OT n=5 rats/group, $F_{(1,8)}$=8.38 p= 0.02). We also observed increased BDNF protein levels in the hippocampus (**Figure 1J;** BDNF n=5-7 rats/F1 group, $F_{(1,10)}$= 4.52, p= 0.05) and reduced mGluR2/3 protein expression in the hippocampus (**Figure 1K;** mGluR2/3 n= 6-7 rats/group, *PRS effect*, $F_{(1,11)}$=11.43 p= 0.006), as well as mRNA transcript levels of mGluR2 and mGluR3 genes in PRS offspring, **(Figure 1L;** n=15 rats/F1 group, *stress effec*t, Grm2 mRNA gene, $F_{(1,28)}$=4.49, p= 0.04; Grm3 mRNA $F_{(1,28)}$=13.03, p=0.0011). In association with the enhanced stress response, F1 PRS males also displayed their characteristic reduction in risk-taking behavior in the EPM, as measured by the reduced time spent in the open arm compared to controls (**Figure 1M**; n=7 rats/F1 group, *stress effec*t, $F_{(1,12\ )}$=17.52, p= 0.001).

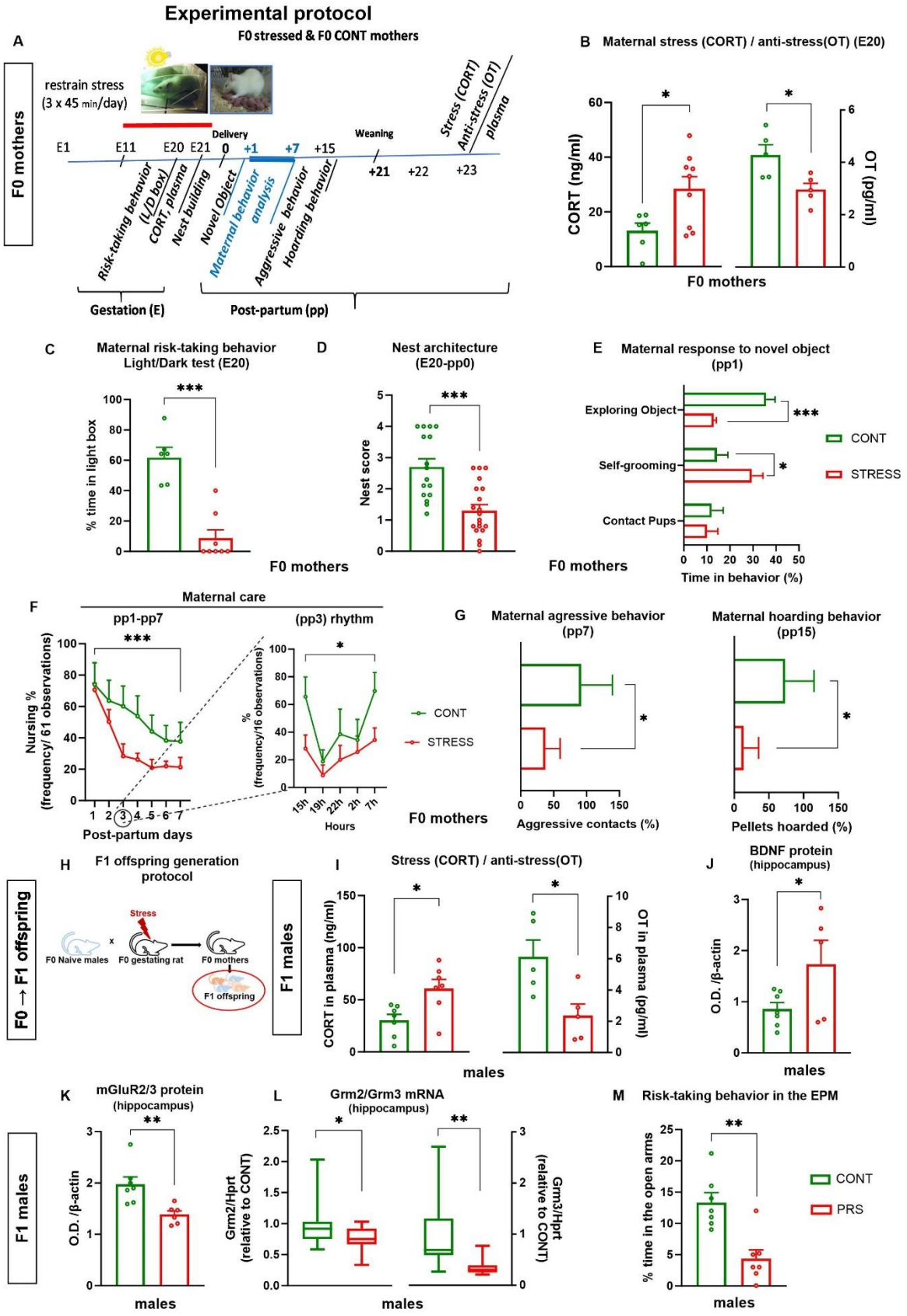


**Figure 1. Gestational stress reduced mother's behavior and stress-antistress balance in F0 females and modulated stress transmission to F1 PRS offspring A**) Experimental design for the analysis of stress/anti-stress balance and behavioral parameters during gestation and the postpartum period in F0 stressed (STRESS) and

control (CONT) mothers. During gestation (E20), F0 stressed mothers displayed **B**) increased corticosterone (CORT) and reduced oxytocin (OT) levels, **C**) reduced risk-taking behavior in the light/dark box, and **D**) poor nest quality. During the postpartum period, stressed mothers presented **E**) an avoidance behavior towards new object in the home cage, **F)** a reduced amount of nursing behavior as well as **G**) poor aggressive behavior toward a male intruder and pellet hoarding behavior. We then assessed F1 adult PRS and CONT unstressed F1 offspring for stress/anti-stress response and molecular and behavioral correlates **H**). PRS impaired in males the stress/anti-stress response **I**) increased CORT and reduced OT levels in response to stress, in association with **J**) increased BDNF protein levels and reduced levels of **K**) mGluR2/3 protein and of **L**) Grm2 and Grm3 mRNA in the hippocampus. **M**) Behaviorally, PRS male rats displayed a reduced risk-taking behavior in the EPM. All values are means ± S.E.M. (n=5-20 rats/group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for CONT *vs*. STRESS. postpartum: pp; Embryonic day: E.

**Like-mother like-daughter transmission of maternal stress and to F2 generation**

We confirmed the programming induced by early maternal stress also on virgin F1 PRS females (adult female offspring of F0 mothers). Indeed, PRS F1 females exhibited the same impairment in the stress/anti-stress balance as PRS males (**Figure 2A**), with higher CORT and lower OT plasma levels after exposure to 30-minute novelty stress (*PRS effect*, CORT n=6-7 rats/F1 group, $F_{(1,11)}=6.45$, p=0.02; OT n=5-6 rats/group, $F_{(1,9)}=6.94$, p=0.02). However, they did not display changes in protein levels of BDNF (**Figure 2B**) in the hippocampus (n=6 rats/F1 group, *PRS effect*, $F_{(1,10)}=0.12$, p=0.73, ns). When assessed for risk-taking behavior in the EPM (**Figure 2C**), PRS females displayed higher exploration of the open arm than the control unstressed group (n=6 rats/F1 group, *PRS effect* $F_{(1,10)}=14.24$, p=0.004), an opposite pattern as compared to F1 PRS males, thus confirming the sex-dimorphic profile in risk-taking behavior induced by PRS, as previously reported (Verhaeghe et al., 2021; Zuena et al., 2008).

Then, the maternal line was set up to evaluate the transmission of maternal stress to F1 mothers (primiparous daughters of F0) (**Figure 2D**). We assessed maternal care, response to a novel object, and the stress/anti-stress balance in plasma in two-month-old F1 females generated by stressed or control F0 mothers (PRS and control F1 mothers, respectively) and mated with F1 males generated by control F0 mothers. Despite neither PRS nor control unstressed F1 dams being exposed to gestational stress, their behavioral profile was identical to that of stressed mothers. Indeed, as for F0 mothers, PRS F1 mothers showed the same avoidance profile to the novel object in the cage at pp1 (**Figure 2E)**, with reduced exploration, increased time spent in self-grooming as well as a reduced contact with pups in the home cage compared to control unstressed F1 dams (n=8 dams/F1 group, *PRS effect*, % of time spent exploring object, $F_{(1,14)}=4.69$, p=0.04); % time self-grooming $F_{(1,14)}= 10.26$, p=0.006; contact with pups $F_{(1,14)}= 5.12$, p=0.04). Remarkably, PRS F1 dams also displayed the same reduction in the amount of maternal care as F0 stressed dams (**Figure 2F**; n=7-8 dams/F1 group, *PRS effect*, $F_{(1,13)}=7.20$, p=0.02) and exhibited a flattened rhythm of nursing behavior during the day and night cycle on pp3 (**Figure 2F**; *PRS effect*, $F_{(1,13)}=16.63$, p=0.001). This indicated that the impairment in maternal behavior induced by gestational stress in F0 dams was intergenerationally transmitted. Consistently, linear regression analysis (**Figure 2G)** showed a significant positive correlation between the nursing behavior of all F0

and F1 dams (r=0.65, p=0.007). We then assessed basal levels of CORT and OT in F1 mothers under stress-elicited conditions **(Figure 2H)**, specifically 5 days after weaning of the pups (pp26). Consistent with the findings in stressed F0 mothers during gestation, PRS F1 dams exhibited the similar imbalance in stress/anti-stress plasma levels with increased CORT and reduced OT (*PRS effect*, n=5 dams/F1 group; CORT, $F_{(1,8)}$=6.70, p=0.03; OT, $F_{(1,8)}$=8.15, p=0.02).

Based on the profile of the F1 PRS mothers, we anticipated the transmission of the programmed phenotype induced by maternal stress to the F2 generation. Indeed, in PRS F2 offspring, we observed the same alterations in the stress/anti-stress balance seen in F1, along with changes in BDNF and glutamate levels in males and the maintenance of the sex-dimorphic profile in the EPM.

Specifically, both PRS F2 male (**Figure 2I)** and female (**Figure 2J)** offspring presented higher CORT and lower OT plasma levels (*PRS effect* F2 males, CORT: n=8 rats/F2 group, $F_{(1,14)}$=5.83, p=0.02; OT: n=10 rats/F2 group, $F_{(1,18)}$=4.93, p=0.03; *PRS effect* F2 females, CORT: n=5-6 rats/group, $F_{(1,9)}$=5.055, p=0.05; OT: n=6-9 rats/F2 group, $F_{(1,13)}$=6.56, p=0.02). As with the F1 PRS generation, BDNF protein expression in the hippocampus was also increased in PRS F2 males with no effect in PRS F2 females (**Figure 2K**; *PRS effect*, n=6 rats/F2 group, males: $F_{(1,10)}$=17.86, p=0.001; females: $F_{(1,10)}$=2.11, p=0.17, n.s.). Additionally, the glutamatergic transmission profile (**Figure 2L)**, in PRS male F2 offspring mirrored that of the F1 generation, showing reduced mGluR2/3 protein and mGlu2 and mGlu3 mRNA transcript levels (**Figure 2L**; *PRS effect*, mGluR2/3 protein: n=6 rats/F2 group, $F_{(1,10)}$=5.36, p=0.04; Grm2 mRNA: n=5-6 rats/F2 group, $F_{(1,9)}$=5.48, p=0.04; Grm3 mRNA: n=5-6 rats/F2 group, $F_{(1,9)}$=5.62, p=0.04). Behaviorally, F2 males explored the open arm of the EPM less, indicating reduced risk-taking behavior, similar to PRS F1 males (**Figure 2M)**. Conversely, F2 PRS females displayed the opposite profile, like PRS F1 females (**Figure 2M**; *PRS effect*, males: n=7 rats/F2 group, $F_{(1,12)}$=13.67, p=0.003; females: n=5-8 rats/F2 group, $F_{(1,11)}$=5.16, p=0.044). Thus, we confirmed the intergenerational programming of maternal stress from the F0 to the F2 generation.

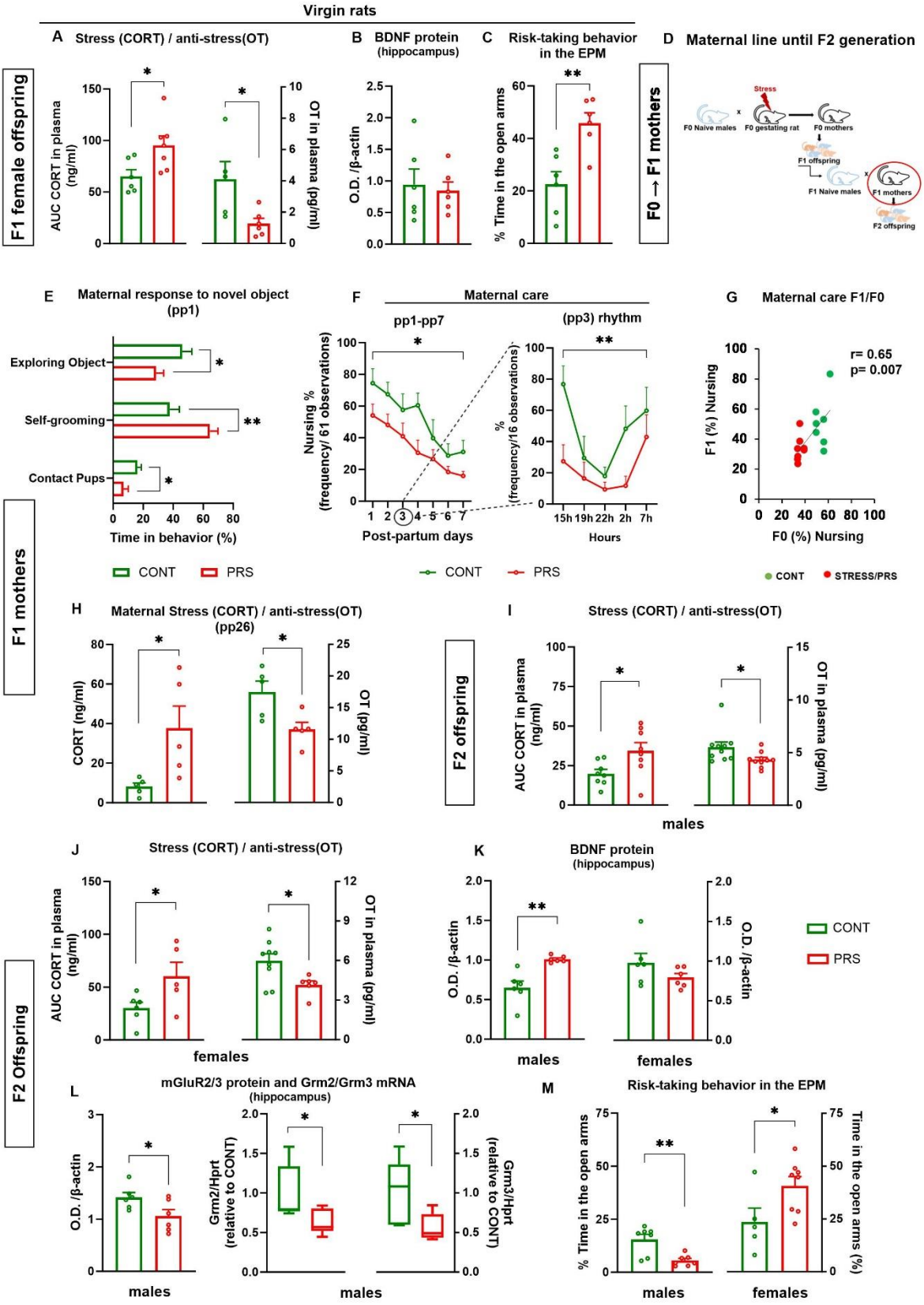


**Figure 2. Like-mother like-daughter transmission of maternal stress up to F2 generation. A**) PRS F1 virgin females displayed increased CORT and reduced OT levels in the plasma, **B**) with no changes in hippocampal BDNF protein expression. **C**) This stress/anti-stress imbalance was associated with increased risk-taking behavior in the EPM, an opposite profile to F1 PRS males. **D**) The protocol for generating F2 offspring

through the maternal line, starting with F0 dams and proceeding to F2 offspring *via* F1 mothers. **E**) In the analysis of maternal response to novel object (pp1), PRS F1 mothers showed a reduced time in contact with pups and exploration of novel object, with increased time of self-grooming. **F**) During the 1st week of pp period (pp1-pp7), F1 PRS mothers displayed reduced maternal care, and on pp3 (inset), PRS also flattened the rhythm of maternal behavior during the day and night cycle. **G**) Maternal nursing behavior in F0 and F1 mothers presented a robust correlation analyzed with Pearson's correlation test. **H**) At pp 26, F1 PRS mothers displayed an imbalance in stress/anti-stress with increased plasmatic CORT and reduced OT levels as the F0 stressed dams. **I-L**) Maternal behavior of PRS F1 dams programmed the phenotype of the F2 offspring. Indeed, PRS increased CORT and reduced OT levels in the plasma in both sexes of F2 generation accompanied with an increase in BDNF protein expression in the hippocampus of males with no changes in females. Moreover, F2 PRS males showed reduced mGluR2/3 protein expression and reduced Grm2 and Grm3 mRNA in the hippocampus. **M**) The stress/anti-stress imbalance of F2 offspring of both sexes was associated with the maintenance of the sex-dimorphic profile in risk-taking behavior (EPM) observed in F1 PRS generation, with PRS males displaying a reduction in the time spent in open arms and PRS females an increase. All values are means ± S.E.M. (n=5-10 rats/group), * $p < 0.05$ and ** $p < 0.01$ for CONT *vs.* STRESS and for CONT *vs.* PRS.

**Evidence of epigenetic intergenerational transmission of maternal stress in F1 and F2 male generation**

We investigated the epigenetic nature of the intergenerational programming induced by reduced maternal care and impaired stress/anti-stress balance in F0 and F1 mothers by focusing on DNA methylation in the hippocampus of F1 and F2 male offspring. A methylation analysis performed by LUMA on the whole hippocampus of the F1 offspring (**Figure 3A**) revealed increased methylation in PRS F1 males compared to the control unstressed group (n= 6 rats/F1 group, *PRS effect*, $F_{(1,10)}=7.45$ p= 0.02). We then focused on key actors of the stress response as Nrc2 (MR) and Nrc1 (GR), and Bdnf in the hippocampus and measured CPG methylation by pyrosequencing along with the mRNA expression. PRS F1 individuals displayed increased methylation in the promoter of Nrc2 (**Figure 3B)** and Nrc1 (**Figure 3D)** genes whereas a reduced methylation of the Bdnf gene promoter (**Figure 3F)** (methylation F1; *PRS effect*, n= 6 rats/F1 group, Nrc2 (MR), $F_{(1,10)}=5.97$ p= 0.03; Nrc1 (GR), $F_{(1,10)}=7.89$ p= 0.018; Bdnf, $F_{(1,10)}=6.64$ p= 0.03). This was in close agreement with the observed changes in the corresponding mRNA transcripts, which were respectively reduced in MR (**Figure 3C)** and GR (**Figure 3E)** while increased in Bdnf (**Figure 3G)** (*PRS effect*, MR, n=13 rats/F1 group, $F_{(1,24)}=4.47$ p= 0.04; GR, n= 5 rats/F1 group, *PRS effect*, $F_{(1,8)}=16.25$ p= 0.003; Bdnf n= 14 rats/group, $F_{(1,26)}=6.65$ p= 0.015). The same epigenetic profile was shared by the F2 generation both at the level of methylation and mRNA transcripts, with a profile of hypermethylation in MR (**Figure 3H)** and GR (**Figure 3J)** and hypomethylation in Bdnf (**Figure 3L)** associated with downregulation for MR (**Figure 3I)** and GR (**Figure 3K)** and upregulation of Bdnf mRNA (**Figure 3M)** observed in PRS F2 rats (methylation *PRS effect*, n= 6 rats/F2 group, MR, $F_{(1,10)}=15.59$ p= 0.002; Nrc1 GR $F_{(1,10)}=9.63$ p= 0.011; Bdnf $F_{(1,10)}=5.06$ p= 0.04; mRNA transcripts *PRS effect*, MR, n= 6-7 rats/F2 group, $F_{(1,11)}=7.28$ p= 0.02; Nrc1 GR n=5 rats/group, $F_{(1,8)}=14.97$ p=0.004; Bdnf n=6-7 rats/group, $F_{(1,11)}=6.65$ p= 0.02). Thus, we confirmed the epigenetic transmission of the intergenerational programming induced by gestational stress in F0 up to F2.

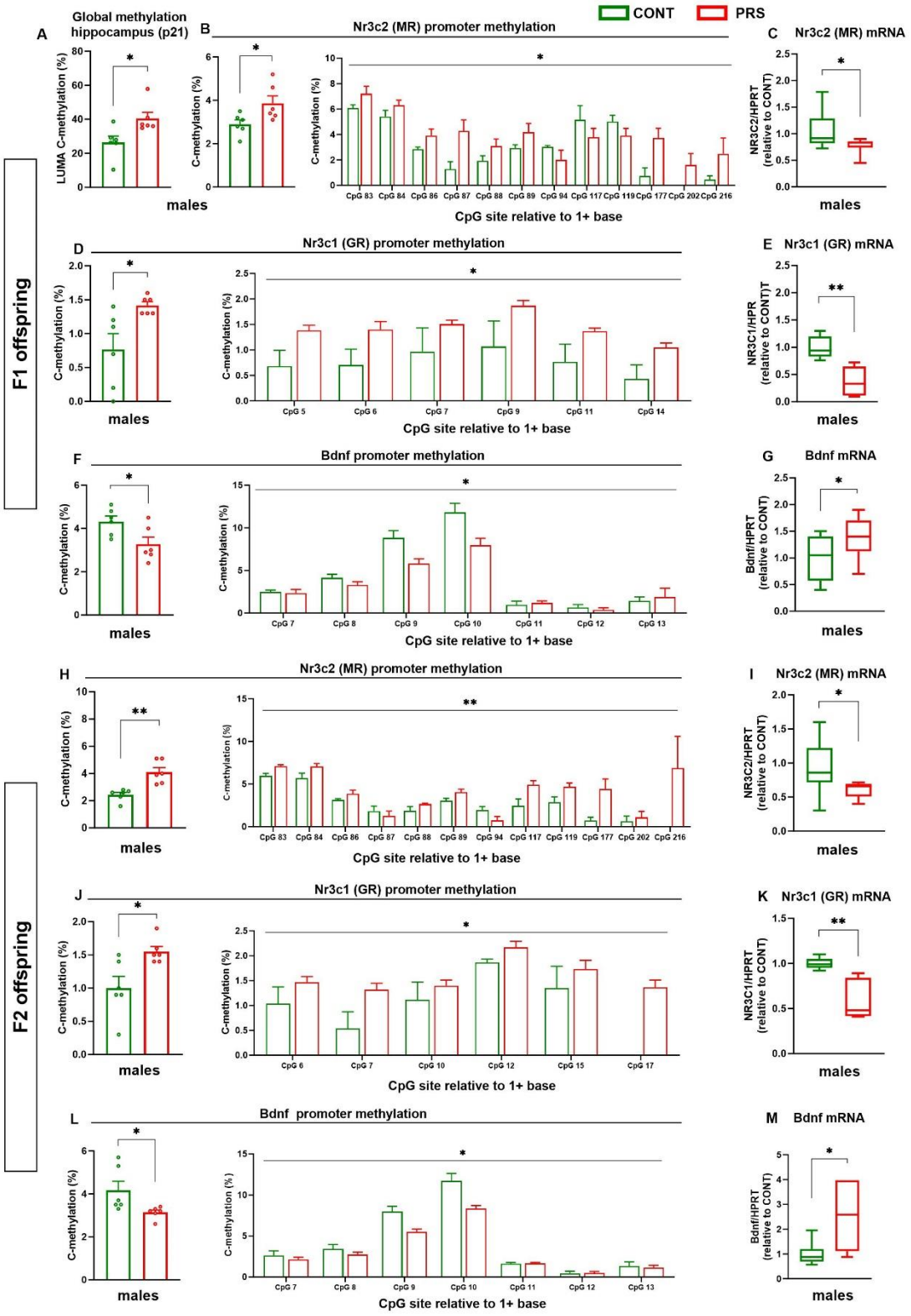


**Figure 3. Evidence of epigenetic intergenerational transmission of maternal stress in F1 and F2 male generation** Epigenetic analyses in F1 and F2 male PRS and CONT rat offspring in the hippocampus revealed **A**) a global profile of hypermethylation in the whole hippocampus of PRS F1 males associated with hypermethylation in **B)** MR (Nr3c2) and **D)** GR (Nr3c1) gene promoters and hypomethylation in **F**) Bdnf gene promoter. A similar profile was observed in F2 PRS individuals (**H, J** and **L**). Consistently with the profile of methylation, MR and GR mRNA transcript were downregulated in both PRS F1 (**C,E**) and PRS F2 (**I,K**) generations, while Bdnf gene expression was increased in both F1 (**G**) and F2 (**M**) PRS individuals. All values are means ± S.E.M. (n=5-7 rats/group), *p < 0.05 and **p < 0.01 for CONT *vs.* PRS F1 and F2 male offspring.

**Like-mother like-daughter transmission of maternal stress can be reversed by maternal oxytocin activation**

An experiment investigating the involvement of oxytocin in the intergenerational transmission of maternal stress used stressed F0 dams treated with the brain-permeant oxytocin receptor agonist, carbetocin (Cbt, 1 mg/kg, i.p.), daily from pp1 to pp7 as illustrated in **Figure 4A**. We then analyzed the impact of postpartum Cbt on maternal behaviors and the stress/anti-stress balance in F0 mothers and evaluated its transmission to F1 mothers. Notably, maternal stress compromised the efficacy of gestation by elevating the relative frequency of abortions out of the total number of abortions recorded (83% stressed vs. 17% control unstressed; 6 abortions n=8-20 dams/F0 group (**Figure 4B**). This impairment was transmitted to F1 daughters/mothers (75% PRS *vs.* 25% control unstressed; 4 abortions, n=8-10 dams/F1 group). However, after F0 maternal treatment with Cbt, abortions were absent in the Cbt-treated PRS F1 dams (**Figure 4B**). As previously observed (see also Gatta et al., 2018) postpartum Cbt administration enhanced maternal behavior in PRS F0 dams and remarkably, we observed the same profile in F1 mothers (**Figure 4C**; *cbt effect*, n=5-6 dams/F0 group, $F_{(2,13)}$=23.01 p=0.00005; n=5-9 dams/F1 group, $F_{(2,17)}$=21.32 p=0.000023). Altogether, maternal care in F1-Cbt treated groups was strongly predicted by maternal care in the F0 mothers as shown by the positive correlation between amount of nursing behavior in F1 and F0 mother groups (**Figure 4D;** r=0.58, p=0.006). The reversal effect of Cbt in F0 stressed dams was also observed in the lowered threshold for licking of the pups following a 15-minute separation on pp7 (**Figure 4E**) (n=5-6 dams/F0 group, *treatment effect*, $F_{(2,13)}$=6.91 p=0.009). Again, postpartum Cbt treatment in stressed F0 dams transmitted the normalization of maternal responsivity to pups' separation in PRS F1 dams (**Figure 4E**; n=6-9 dams/F1 group, *Cbt effect*, $F_{(2,17)}$=9.69 p=0.0015) which displayed decreased latency to lick the pups (**Figure 4D**). Interestingly, maternal responsivity to pups' separation was predicted by the amount of nursing behavior. Indeed, correlation analysis within each mother generation indicated that a reduced maternal care predicted an increased latency to lick pups after separation (F0, r=-0.53, p=0.01; F1, r=-0.68, p=0.0009), with the stressed/PRS group and Cbt-treated groups being clustered in opposite directions. Moreover, maternal Cbt in F0 corrected the stress/anti-stress balance in F0 stressed dams by reducing CORT (**Figure 4G**) and increasing OT plasma levels compared to control unstressed F0 dams (**Figure 4I**). Again, this profile was transmitted to the F1 generation of Cbt-treated PRS dams, which displayed reduced CORT and enhanced OT plasma levels comparable to control unstressed F1 dams (**Figure 4G**; *Cbt effect* CORT F0, n=5 dams/F0 group, $F_{(2,12)}$=10.35, p=0.002; CORT F1, n=5-6 dams/F1 group, $F_{(2,13)}$=9.87, p=0.002; **Figure 4I;** OT F0, n=5-6 dams/F0 group, $F_{(2,13)}$=4.37 p=0.035; OT F1, n=5-9 dams/F1 group, $F_{(2,17)}$=3.91, p=0.039). In accordance with a central role of the stress/anti-stress balance in regulating maternal behavior both in F0 and F1 mother we reported that CORT levels in F1 were positively associated to CORT in F0 (**Figure 4H**; r=0.55, p=0.03) and that OT was strongly associated with maternal reactivity in F0 (**Figure 4J**; negative correlation, r=-0.53, p=0.03) while with maternal

care in F1 (**Figure 4J**; positive correlation, r=0.43, p=0.05). In all significant associations identified, Stress/PRS + Veh and Stress /PRS +Cbt groups were distinctly clustered in opposite directions on the scatterplot, with the Cbt group overlapping distribution of the CONT group, thus indicating the restorative effect to control unstressed levels of Cbt. Altogether, we confirmed the role of the stress/anti-stress balance in the intergenerational transmission of reduced maternal behavior induced by gestational stress in F0.

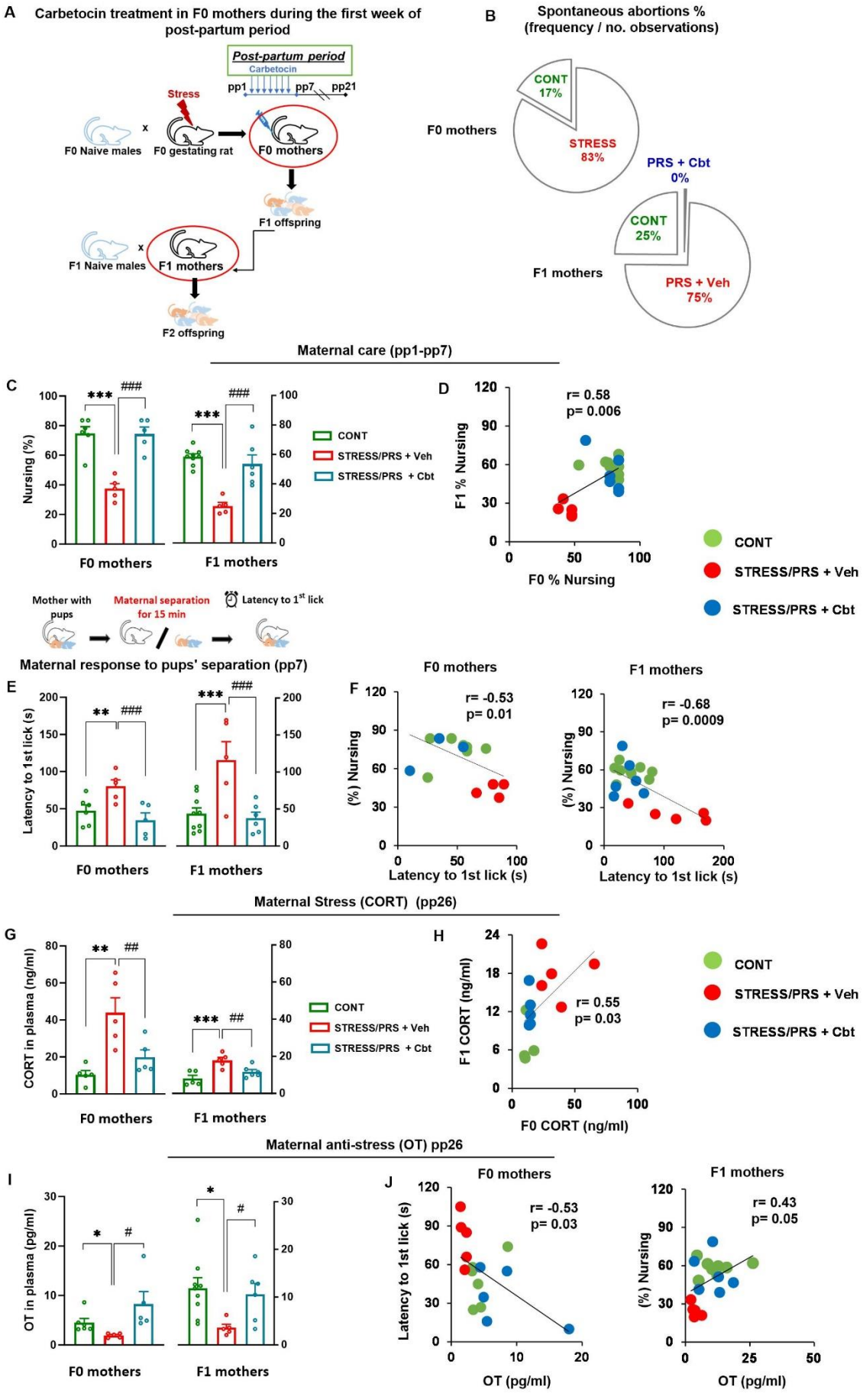
A
Carbetocin treatment in F0 mothers during the first week of post-partum period
Post-partum period
Carbetocin
pp1
pp7
pp21
Stress
F0 Naive males
F0 gestating rat
F0 mothers
F1 offspring
F1 Naive males
F1 mothers
F2 offspring
B
Spontaneous abortions %
(frequency / no. observations)
F0 mothers
CONT 17%
STRESS 83%
F1 mothers
PRS + Cbt 0%
CONT 25%
PRS + Veh 75%
Maternal care (pp1-pp7)
C
Nursing (%)
F0 mothers
F1 mothers
CONT
STRESS/PRS + Veh
STRESS/PRS + Cbt
D
r= 0.58
p= 0.006
F1 % Nursing
F0 % Nursing
CONT
STRESS/PRS + Veh
STRESS/PRS + Cbt
Mother with pups
Maternal separation for 15 min
Latency to 1st lick
Maternal response to pups' separation (pp7)
E
Latency to 1st lick (s)
F0 mothers
F1 mothers
F
F0 mothers
r= -0.53
p= 0.01
(%) Nursing
Latency to 1st lick (s)
F1 mothers
r= -0.68
p= 0.0009
(%) Nursing
Latency to 1st lick (s)
Maternal Stress (CORT) (pp26)
G
CORT in plasma (ng/ml)
F0 mothers
F1 mothers
CONT
STRESS/PRS + Veh
STRESS/PRS + Cbt
H
r= 0.55
p= 0.03
F1 CORT (ng/ml)
F0 CORT (ng/ml)
CONT
STRESS/PRS + Veh
STRESS/PRS + Cbt
Maternal anti-stress (OT) pp26
I
OT in plasma (pg/ml)
F0 mothers
F1 mothers
J
F0 mothers
r= -0.53
p= 0.03
Latency to 1st lick (s)
OT (pg/ml)
F1 mothers
r= 0.43
p= 0.05
(%) Nursing
OT (pg/ml)

**Figure 4. Like-mother like-daughter transmission of maternal stress can be reversed by maternal oxytocin activation A)** Experimental design of the carbetocin (Cbt) postpartum treatment in F0 mothers and the generation of F1 mothers through the maternal line. **B)** Gestational Stress induced an increase of spontaneous abortions in F0 which was transmitted to PRS F1 dams. However, this deficit was not present in PRS F1 dams descending stressed-Cbt treated mothers. **C)** Maternal care was reduced by gestational stress in F0 and F1 mothers and Cbt treatment increased nursing behavior up to F1 dams, as evidenced by **D)** positive correlation between F0 and F1 maternal care. **E)** In response to a brief pups' separation both F0 stressed and F1 PRS dams showed an increased latency for first contact with the pups, which was intergenerationally rescued by Cbt treatment. **F)** Reduced nursing behavior predicted an impaired responsivity to pups' separation within each generation; **G)** Maternal Cbt in F0 corrected in both F0 and F1 stressed or PRS dams the stress/anti-stress balance by **G**) reducing CORT and **I**) increasing OT plasma levels compared to control unstressed dams. **H)** Intergenerational transmission of CORT levels and correction by Cbt as evidenced by the positive correlations between F0 and F1 **J)** OT levels correlated with maternal behaviors in F0 and F1 dams respectively. All values are means ± S.E.M. (n=5-9 rats/group), *p < 0.05, **p < 0.01 and ***p < 0.001 for CONT *vs.* STRESS/PRS. #p < 0.05; ##p < 0.01and ###p < 0.001 for STRESS/PRS + Veh vs STRESS/PRS + Cbt.

## The postnatal intergenerational inheritance of PRS can be reversed by maternal oxytocin activation

We anticipated that correcting maternal behavior in F0 and F1 would reverse the PRS phenotype in both male and female PRS offspring in F1. We extended our investigation of intergenerational transmission by examining the persistence of carbetocin treatment effects into the F2 generation (**Figure 5A**). Indeed, carbetocin treatment in F0 dams interrupted the intergenerational inheritance of PRS into the F2 generation. F2 PRS males and females exhibited the same correction in stress/anti-stress balance in plasma and gene expression in the hippocampus as seen in the F1 generation, along with a reversal of risk-taking behavior. In particular, F0 maternal carbetocin reduced CORT response to stress in both male and female F1 PRS rats while increasing OT plasma levels (*Cbt effect*, **Figure 5B**; CORT males n=5 rats/F1 group, $F_{(2,12)}$=4.37 p=0.04; CORT females n=5-6 rats/F1 group, $F_{(2,14)}$=6.27, p=0.01; **Figure 5B**; OT males n=5-9 rats/F1 group, $F_{(2,17)}$=7.55, p=0.004; OT females n=6-9 rats/F1 group, $F_{(2,20)}$=5.05 p=0.02) with respect to PRS-Veh treated F1 offspring. The same profile was observed in the PRS-Cbt treated F2 generation of both sexes which resented lower CORT and increased OT levels with respect to PRS-Veh treated F2 animals (*Cbt effect*, **Figure 5D**; CORT males n=9-10 rats/F2group, $F_{(2,26)}$=4.49 p= 0.02; CORT females n=8 rats/F2group, $F_{(2,\ 21)}$=4.72 p= 0.02; **Figure 5E**; OT males, n= 9-10 rats/F2group, $F_{(2,25)}$=4.63 p= 0.02; OT females, n=7 rats/F2group, $F_{(2,18)}$=4.53 p= 0.05). Additionally, we observed intergenerational transmission of Cbt correction at the level of BDNF protein in the hippocampus, with Cbt restoring to control levels the increased levels of BDNF in PRS males in both F1 and F2 generation, without effect on females (**supplementary Figure S3**).

Behaviorally, maternal Cbt treatment in F0 reverted to control unstressed levels the sex-dimorphic profile shown in the PRS F1 offspring in the EPM, with reduced levels of risk-taking behavior in PRS males and opposite profile in PRS females (*Cbt effect*, **Figure 5F-G** males, n=16-17 rats/F1 group, $F_{(2,46)}$; % open arm F=17.20 p= 0.0001; entries to open arm F=13.73, p=0.0001; females n=10-17 rats/F1 group, $F_{(2,39)}$ % time open arm F=5,61 p= 0,007; entries open arm F= 3.43 p=0.04). Again, the identical

profile of correction was intergenerationally transmitted to F2 PRS offspring Cbt-treated animals (*Cbt effect*, **Figure 5I-J**; males: n=10-14 rats/F2 group, $F_{(2,34)}$, % open arm F=24.16 p= 0.0001; entries open arm, F=16.13, p=0.0001; females n=8-14 rats/F2 group, $F_{(2,28)}$, % time open arm F=8,17 p= 0.002; entries to open arm F=4.12, p=0.03).

Moreover, we evidenced an epigenetic intergenerational transmission of PRS along the stress/anti-stress axis, since GR, MR, and OT mRNA transcripts were reduced by PRS in both sexes, as well as in the F1 (**Figure 5L**; *PRS effect*, $_{\text{PRS-Veh } vs. \text{ CONT}}$ Mann-Whitney; n=5 rats /sex/F1 group, F1 males: GR; z = 2.50, p = 0.007; MR z= 2.08, p = 0.031; OT z = 2.50, p = 0.007; F1 females: GR; z = 2.50, p = 0.008; MR z = 2.50, p = 0.008; OT z = 2.51, p = 0.008;) and F2 generations (**Figure 5M**; n=5 rats /sex/F2 group, males: GR; *PRS effect* z = 2.50, p = 0.007; MR z = 2.08, p = 0.031; OT z = 1.88, p = 0.05; F2 females: GR; z = 2.50, p = 0.008; MR z = 2.50, p = 0.007; OT z = 1.88 p = 0.05). However, Cbt restored gene expression to control (unstressed) levels in both generations and sexes, thus demonstrating an epigenetic effect by correcting the gene expression disrupted by PRS (**Figure 5L**; *Cbt effect* $_{\text{PRS-Veh } vs. \text{ PRS Cbt}}$, n=5 rats /sex/F1 group, F1 males: GR; z = 2.29, p=0.015; MR z = 2.08, p=0.031; OT z = 1.88, p=0.05; F1 Females, *Cbt effect*: z = 2.50, p = 0.008; MR, z = 2.30, p=0.02; OT, z = 2.50, p=0.008; and **Figure 5M**; n=5 rats /sex/F2 group, males: GR; *Cbt effect* z = 0.20, p=0.84; MR z= 0.41, p=0.69; OT z = 1.88, p=0.05; F2 females: GR; *Cbt effect* z = 2.29, p = 0.02; MR z = 2.08, p=0.03; OT z= 2.50, p=0.007). Altogether, we demonstrated that the correction of postnatal PRS inheritance was epigenetically transmitted by the F1 mother up to F2 offspring, following oxytocinergic activation in the F0 generation.

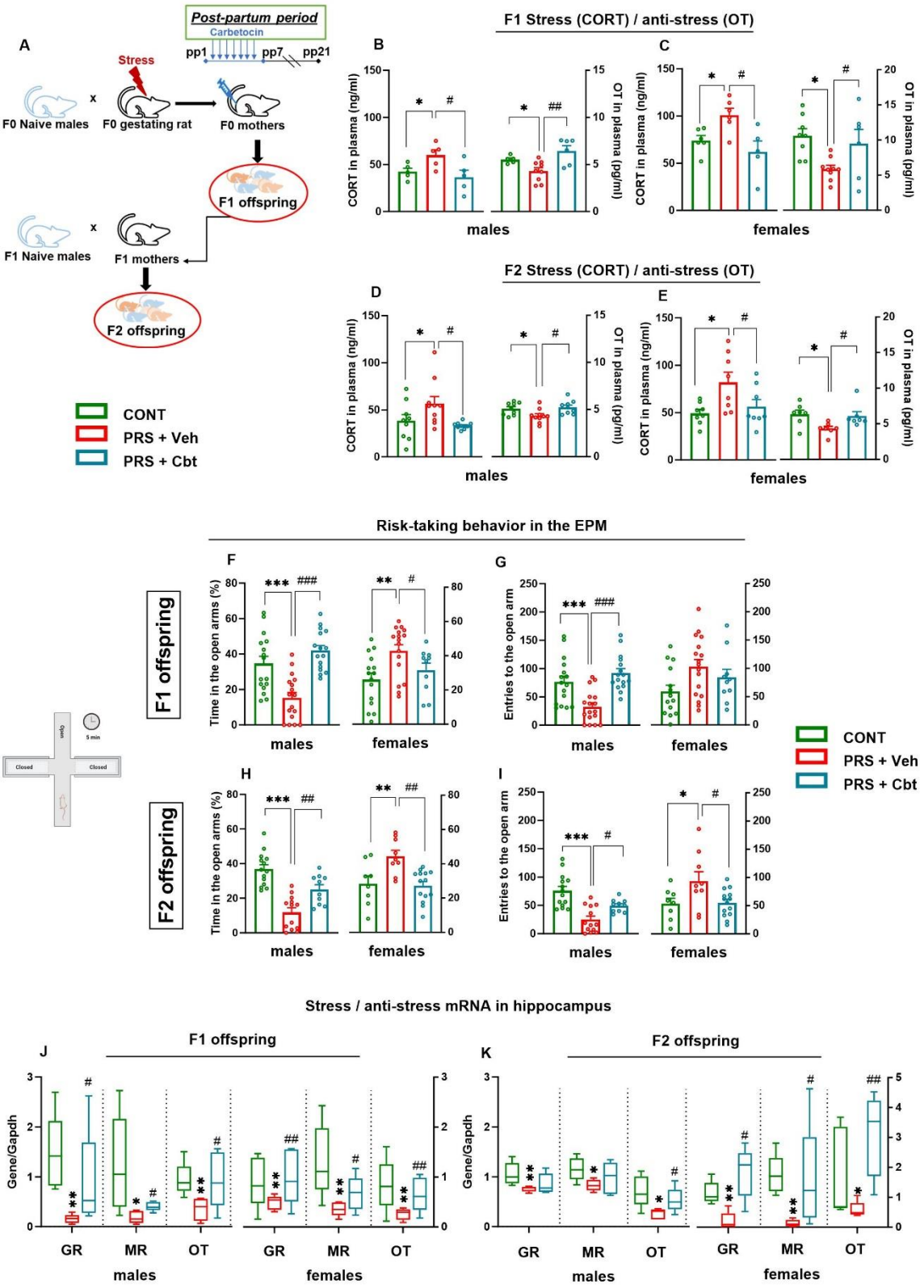


**Figure 5. The postnatal intergenerational inheritance of PRS can be reversed by maternal oxytocin activation A)** We examined whether correcting maternal behavior in F0 and F1 by carbetocin would reverse the PRS phenotype in both male and female PRS offspring in F1, and if the effects persisted up to the F2. **B-E**) The alterations induced by PRS on the stress/anti-stress balance in both male and female F1 and F2 PRS rats were corrected by Cbt with lower CORT response to stress and increased OT plasma levels with respect to PRS-Veh treated offspring; **F-I**) PRS induced a sex-dimorphic profile in the EPM, with reduced levels of risk-taking behavior in PRS males and opposite profile in PRS females, which was reverted to control unstressed levels

following maternal Cbt treatment in F0. PRS effect and Cbt correction were intergenerationally transmitted to F2 PRS offspring; **J-K**) Epigenetic intergenerational transmission of PRS along the stress/anti-stress axis, since GR, MR, and OT mRNA transcripts were reduced by PRS in both the F1 and F2 generations and in both sexes and restored by Cbt to control (unstressed) levels in both generations and sexes. All values are means ± S.E.M. (n=5-10 rats/group), *p < 0.05; **p < 0.01 and ***p < 0.001 for CONT *vs.* STRESS F0 dams. #p < 0.05; ##p < 0.01for PRS vs PRS + Cbt.

## DISCUSSION

Our findings in a perinatal stress rat model revealed the intergenerational transmission of maternal stress and altered maternal care behavior, associated with an impaired stress/anti-stress balance that was epigenetically transmitted across generations through a "like mother, like daughter" mechanism. Remarkably, we provided groundbreaking evidence that postpartum carbetocin (Cbt) treatment in F0 stressed rat mothers reversed this imbalance and associated behavioral and biochemical alterations up to F2 PRS offspring of both sexes. This offers insights into the biological mechanisms of intergenerational stress transmission and identifies maternal oxytocin as a potential therapeutic target to break this cycle.

Gestational stress had a major impact on a mother's adaptation to motherhood, revealing that F0 stressed mothers were poorly equipped to face it. We have previously shown that during lactation and after weaning, stressed mothers displayed reduced body weight gain and remained in a state of enhanced reactivity to stress in novel environments (Darnaudéry et al., 2004a). Multiple adaptations of the mother's neuroendocrine system are needed to successfully adapt to pregnancy, parturition, and lactation, with the balance between glucocorticoids and oxytocin playing a pivotal role (Brunton and Russell, 2008). Here, by focusing on the peripartum period, we evidenced that stress impaired the establishment of proper maternal physiology during gestation, resulting in enhanced stress reactivity and reduced oxytocinergic tone in plasma, associated with reduced environmental exploration. The stress/anti-stress imbalance we observed in the mothers delayed the onset of proper nest-building during the peripartum period, a phenomenon associated with a decline in maternal behavior, similar to non-lactating rats (Rosenblatt, 1975). Other alterations included avoidance behavior toward novel object, poor defensive or hoarding behavior during lactation, and diminished response to pups' separation. Above all, stressed mothers displayed a marked reduction in nursing behavior toward the pups. Strikingly, several of these alterations, such as avoidance behavior toward novelty, reduced response to pups' separation, and reduced maternal care, were observable to the same extent in the F1 PRS dams. Additionally, gestational stress reduced the efficacy of pregnancy, evidenced by a higher frequency of spontaneous abortions in stressed mothers compared to unstressed ones and again this phenomenon was observable in PRS F1 dams. Remarkably, Cbt exerted a general positive effect on the overall health of the dams, reflected not only in enhanced behaviors toward the pups (maternal care and response to pups' separation) but also in the absence of spontaneous abortions in the daughter offspring of Cbt-

treated stressed mothers. Mostly, the beneficial effects of Cbt on the stressed F0 mother were transferred to the F1 PRS daughter.

Our study showed that maternal stress epigenetically programmed the stress response in the offspring up to the F2 generation, identifying the alteration in the stress/anti-stress balance in stress-exposed rat mothers as a key mechanism of intergenerational transmission. Indeed, two striking findings confirmed this mechanistic hypothesis. First, the enhanced CORT/reduced OT profile observed in the F0 mother during gestation persisted in the F1 PRS dam (daughter of F0 stressed dams) at the end of the lactation period, after weaning of pups. Second, by restoring the CORT/OT balance in the F0 mother with postpartum Cbt, the reversal of the PRS phenotype in the F1 daughter was also observed. We have previously shown that Cbt given to the mother can correct the PRS molecular and behavioral phenotype in the F1 male offspring, with long-lasting effects maintained up to aged individuals (Gatta et al., 2018; Morley-Fletcher et al., 2024). Adaptations in the mother's brain and physiology during lactation are maintained by external stimuli from the young (Brunton and Russell, 2008), and parenting experiences activate the oxytocin system, creating a positive feedback loop between oxytocin and parenting behaviors (Nagasawa et al., 2012). Here, we moved beyond the F1 offspring and evidenced a positive oxytocin loop transmitted across generations up to F2, while maternal stress disrupted this process by inducing a negative loop between glucocorticoids and maternal care, which was reduced across F0 and F1 mother's generations. Enhancing maternal behavior through oxytocinergic activation with Cbt, exerted an anti-stress effect on the F0 mother, thereby enhancing F1 offspring's oxytocin levels and parenting activities in adulthood, demonstrating a "like mother, like daughter" effect. Increased oxytocin levels during parenting are also associated with infant attachment behaviors, forming a positive feedback loop of attachment and parenting behaviors in both the infant-mother dyad. Thus, it is likely that a positive feedback loop of parenting and affiliative behaviors could span across generations and contribute to the reversal of the PRS phenotype in F1 PRS mothers and F1 and F2 PRS offspring through the regulation of the CORT/OT balance.

Variations in maternal care have been shown to epigenetically program stress patterns in the offspring (Szyf et al., 2007). Here, we provided evidence that alterations in the amount of nursing behavior as a consequence of gestational stress in F0 and F1 stressed/PRS mothers programmed in the F1 and F2 offspring a profile of hypermethylation of GR and MR gene promoters, associated with downregulation of their respective gene transcripts. Remarkably, changes in gene expression were intergenerationally transmitted in association with the related biochemical and behavioral alterations observed in the F2 PRS generation. Moreover, a hypomethylation profile was observed for the Bdnf gene promoter, associated with increased expression of its transcript and protein in the hippocampal region in PRS rats in both F1 and F2 generations. We have previously reported increased BDNF protein in PRS males in association with impaired neurogenesis in the hippocampus (Zuena et al., 2008), which can be reversed by antidepressant/pharmacological treatment (Morley-Fletcher et al., 2018, 2011), which can be reserved by antidepressant/pharmacological treatment. Specific changes in the methylation profile of

the Bdnf gene promoter have been reported in other models of postnatal stress, such as maternal maltreatment (Roth et al., 2009), with a general profile of hypermethylation in the prefrontal cortex of maltreated individuals, observable in F1 and F2 generations (Roth et al., 2009). Here, in an animal model of perinatal stress (prenatal + postnatal stress), we showed for the first time the epigenetic regulation by PRS of Bdnf via a maternal line. We anticipated that correcting maternal behavior in F0 and F1 would reverse the PRS phenotype in both male and female PRS offspring in F1 and extended our investigation of intergenerational transmission by examining the persistence of carbetocin treatment effects into the F2 generation. F2 PRS males and females exhibited the same correction in stress/anti-stress balance in plasma and gene expression in the hippocampus as seen in the F1 generation, along with a reversal of risk-taking behavior. Postpartum Cbt reverted the gene expression of the stress/anti-stress balance in F1 as previously reported (Gatta et al., 2018), thus sustaining its epigenetic action. Altogether, carbetocin treatment in F0 dams interrupted the intergenerational inheritance of PRS into the F2 generation. To our knowledge, this is the first evidence in an animal model of perinatal stress for an environmental/mother-associated intervention to break the chain of stress transmission.

Another important finding, is that we examined sex differences in PRS transmission across generations. Previously, sex differences in second-generation transmission were poorly investigated, primarily in the paternal line-transmission stress model, where sex-related changes often appeared inconsistent between generations (Franklin et al., 2010). Our findings on maternal line transmission of PRS showed consistent alterations in the stress/anti-stress balance in both PRS males and females. Also, the behavioral and biochemical patterns induced by PRS were identically transmitted to the second generation and were specifically corrected to control unstressed profiles for each sex by Cbt treatment. The consistency of our results obtained through the maternal line supports the positive loop of OT-enhancement of the stress-antistress balance in the mother-infant dyad in the PRS model.

Here, PRS effects resulted in long-term changes in gene expression across two generations of offspring. Although epigenetic programming by maternal care is limited to a critical window of time, its effects are stable (Szyf et al., 2007). However, the epigenome is dynamic and its effects are reversible if the intervention occurs in an appropriate time window. Thus, another notable finding of our study was that transient OT activation with Cbt during the first week of lactation was enough to completely reverse the stressed/PRS phenotype across two generations of mothers and two generations of offspring. One possible explanation for the efficacy of the carbetocin intervention within this short time window, should consider the first week of lactation as the period where the mother-infant bond is strongest. Indeed, studies in the PRS model on adoption and foster care at various lactation stages (Barbazanges et al., 1996b; Darnaudéry et al., 2004b), suggest that there is a sensitive period during which modulations of maternal behavior through fostering can alter the behavior of both pups and dams. Specifically, only early adoption increased maternal licking behavior, which may protect the pups (Barbazanges et al., 1996b). Moreover, early adoption reduced pups' ultrasound emissions in response to stressful separation, whereas later adoptions increased these emissions (Darnaudéry et al., 2004b).

Here, we showed that the first lactation week represents a window of opportunity for interventional strategies that, by targeting the stress/anti-stress balance which regulates maternal behavior, can interrupt the intergenerational transmission of perinatal stress.

In conclusion, our study underscores the significant impact of maternal stress on intergenerational transmission through epigenetic mechanisms. Postpartum carbetocin treatment in stressed rat mothers effectively reversed stress-induced imbalances, demonstrating the potential of maternal oxytocin as a therapeutic target. Restoring the stress/anti-stress balance through oxytocinergic activation not only improved maternal care but also positively influenced subsequent generations, creating a beneficial feedback loop. Our findings highlight the critical window during early lactation for interventions to break the cycle of stress transmission. These insights pave the way for new strategies beyond pharmacological approaches to mitigate the effects of maternal stress on offspring health and development and the health of future generations.

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**REFERENCES**


1) Angelucci, L., Patacchioli, F.R., Scaccianoce, S., Di Sciullo, A., Cardillo, A., Maccari, S., 1985. A model for later-life effects of perinatal drug exposure: maternal hormone mediation. Neurobehav. Toxicol. Teratol. 7, 511–517.

2) Barbazanges, A., Piazza, P.V., Moal, M.L., Maccari, S., 1996a. Maternal Glucocorticoid Secretion Mediates Long-Term Effects of Prenatal Stress. J. Neurosci. 16, 3943–3949. https://doi.org/10.1523/JNEUROSCI.16-12-03943.1996

3) Barbazanges, A., Vallée, M., Mayo, W., Day, J., Simon, H., Moal, M.L., Maccari, S., 1996b. Early and Later Adoptions Have Different Long-Term Effects on Male Rat Offspring. J. Neurosci. 16, 7783–7790. https://doi.org/10.1523/JNEUROSCI.16-23-07783.1996

4) Boscardin, C., Manuella, F., Mansuy, I.M., 2022. Paternal transmission of behavioural and metabolic traits induced by postnatal stress to the 5th generation in mice. Environ. Epigenetics 8, dvac024. https://doi.org/10.1093/eep/dvac024

5) Brunton, P.J., Russell, J.A., 2008. The expectant brain: adapting for motherhood. Nat. Rev. Neurosci. 9, 11–25. https://doi.org/10.1038/nrn2280

6) Champagne, F., Diorio, J., Sharma, S., Meaney, M.J., 2001. Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proc. Natl. Acad. Sci. U. S. A. 98, 12736–12741. https://doi.org/10.1073/pnas.221224598

7) Champagne, F.A., Meaney, M.J., 2006. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. Biol. Psychiatry 59, 1227–1235. https://doi.org/10.1016/j.biopsych.2005.10.016

8) Darnaudéry, M., Dutriez, I., Viltart, O., Morley-Fletcher, S., Maccari, S., 2004a. Stress during gestation induces lasting effects on emotional reactivity of the dam rat. Behav. Brain Res. 153, 211–216. https://doi.org/10.1016/j.bbr.2003.12.001

9) Darnaudéry, M., Koehl, M., Barbazanges, A., Cabib, S., Moal, M., Maccari, S., 2004b. Early and Later Adoptions Differently Modify Mother-Pup Interactions. Behav. Neurosci. 118, 590–6. https://doi.org/10.1037/0735-7044.118.3.590

10) Eriksson, M., Räikkönen, K., Eriksson, J.G., 2014. Early life stress and later health outcomes—findings from the Helsinki Birth Cohort Study. Am. J. Hum. Biol. 26, 111–116. https://doi.org/10.1002/ajhb.22502

11) Francis, D.D., Champagne, F.A., Liu, D., Meaney, M.J., 1999. Maternal care, gene expression, and the development of individual differences in stress reactivity. Ann. N. Y. Acad. Sci. 896, 66–84. https://doi.org/10.1111/j.1749-6632.1999.tb08106.x

12) Franklin, T.B., Russig, H., Weiss, I.C., Gräff, J., Linder, N., Michalon, A., Vizi, S., Mansuy, I.M., 2010. Epigenetic transmission of the impact of early stress across generations. Biol. Psychiatry 68, 408–415. https://doi.org/10.1016/j.biopsych.2010.05.036

13) Gatta, E., Mairesse, J., Deruyter, L., Marrocco, J., Van Camp, G., Bouwalerh, H., Lo Guidice, J.-M., Morley-Fletcher, S., Nicoletti, F., Maccari, S., 2018. Reduced maternal behavior caused by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance. NeuroToxicology 66, 138–149. https://doi.org/10.1016/j.neuro.2018.04.005

14) Karimi, M., Johansson, S., Ekström, T.J., 2006. Using LUMA: a Luminometric-based assay for global DNA-methylation. Epigenetics 1, 45–48. https://doi.org/10.4161/epi.1.1.2587

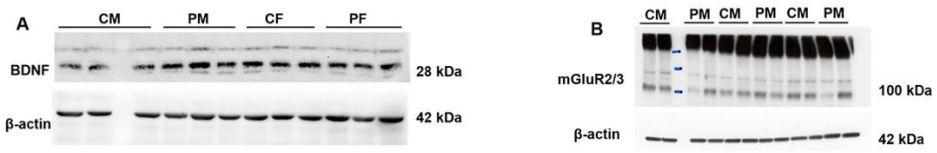
15) Klengel, T., Dias, B.G., Ressler, K.J., 2016. Models of Intergenerational and Transgenerational Transmission of Risk for Psychopathology in Mice. Neuropsychopharmacology 41, 219–231. https://doi.org/10.1038/npp.2015.249

16) Maccari, S., Krugers, H.J., Morley-Fletcher, S., Szyf, M., Brunton, P.J., 2014. The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. J. Neuroendocrinol. 26, 707–723. https://doi.org/10.1111/jne.12175
17) Maccari, S., Piazza, P.V., Kabbaj, M., Barbazanges, A., Simon, H., Le Moal, M., 1995. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J. Neurosci. Off. J. Soc. Neurosci. 15, 110–116.
18) Maccari, S., Polese, D., Reynaert, M.-L., Amici, T., Morley-Fletcher, S., Fagioli, F., 2017. Early-life experiences and the development of adult diseases with a focus on mental illness: The Human Birth Theory. Neuroscience 342, 232–251. https://doi.org/10.1016/j.neuroscience.2016.05.042
19) Marrocco, J., Mairesse, J., Ngomba, R.T., Silletti, V., Van Camp, G., Bouwalerh, H., Summa, M., Pittaluga, A., Nicoletti, F., Maccari, S., Morley-Fletcher, S., 2012. Anxiety-Like Behavior of Prenatally Stressed Rats Is Associated with a Selective Reduction of Glutamate Release in the Ventral Hippocampus. J. Neurosci. 32, 17143–17154. https://doi.org/10.1523/JNEUROSCI.1040-12.2012
20) Marrocco, J., Verhaeghe, R., Bucci, D., Di Menna, L., Traficante, A., Bouwalerh, H., Van Camp, G., Ghiglieri, V., Picconi, B., Calabresi, P., Ravasi, L., Cisani, F., Bagheri, F., Pittaluga, A., Bruno, V., Battaglia, G., Morley-Fletcher, S., Nicoletti, F., Maccari, S., 2020. Maternal stress programs accelerated aging of the basal ganglia motor system in offspring. Neurobiol. Stress 13, 100265. https://doi.org/10.1016/j.ynstr.2020.100265
21) Morley-Fletcher, S., Gaetano, A., Gao, V., Gatta, E., Van Camp, G., Bouwalerh, H., Thomas, P., Nicoletti, F., Maccari, S., 2024. Postpartum Oxytocin Treatment via the Mother Reprograms Long-Term Behavioral Disorders Induced by Early Life Stress on the Plasma and Brain Metabolome in the Rat. Int. J. Mol. Sci. 25, 3014. https://doi.org/10.3390/ijms25053014
22) Morley-Fletcher, S., Mairesse, J., Soumier, A., Banasr, M., Fagioli, F., Gabriel, C., Mocaer, E., Daszuta, A., McEwen, B., Nicoletti, F., Maccari, S., 2011. Chronic agomelatine treatment corrects behavioral, cellular, and biochemical abnormalities induced by prenatal stress in rats. Psychopharmacology (Berl.) 217, 301–313. https://doi.org/10.1007/s00213-011-2280-x
23) Morley-Fletcher, S., Zuena, A.R., Mairesse, J., Gatta, E., Van Camp, G., Bouwalerh, H., Riozzi, B., Battaglia, G., Pittaluga, A., Olivero, G., Mocaer, E., Bretin, S., Nicoletti, F., Maccari, S., 2018. The reduction in glutamate release is predictive of cognitive and emotional alterations that are corrected by the positive modulator of AMPA receptors S 47445 in perinatal stressed rats. Neuropharmacology 135, 284–296. https://doi.org/10.1016/j.neuropharm.2018.03.018
24) Nagasawa, M., Okabe, S., Mogi, K., Kikusui, T., 2012. Oxytocin and mutual communication in mother-infant bonding. Front. Hum. Neurosci. 6, 31. https://doi.org/10.3389/fnhum.2012.00031
25) Rosenblatt, J.S., 1975. Prepartum and postpartum regulation of maternal behaviour in the rat. Ciba Found. Symp. 17–37. https://doi.org/10.1002/9780470720158.ch3
26) Roth, T.L., Lubin, F.D., Funk, A.J., Sweatt, J.D., 2009. LASTING EPIGENETIC INFLUENCE OF EARLY-LIFE ADVERSITY ON THE BDNF GENE. Biol. Psychiatry 65, 760–769. https://doi.org/10.1016/j.biopsych.2008.11.028
27) Szyf, M., Weaver, I., Meaney, M., 2007. Maternal care, the epigenome and phenotypic differences in behavior. Reprod. Toxicol. Elmsford N 24, 9–19. https://doi.org/10.1016/j.reprotox.2007.05.001
28) Toepfer, P., O'Donnell, K.J., Entringer, S., Garg, E., Heim, C.M., Lin, D.T.S., MacIsaac, J.L., Kobor, M.S., Meaney, M.J., Provençal, N., Binder, E.B., Wadhwa, P.D., Buss, C., 2019. Dynamic DNA methylation changes in the maternal oxytocin gene locus (OXT) during pregnancy predict postpartum maternal intrusiveness. Psychoneuroendocrinology 103, 156–162. https://doi.org/10.1016/j.psyneuen.2019.01.013

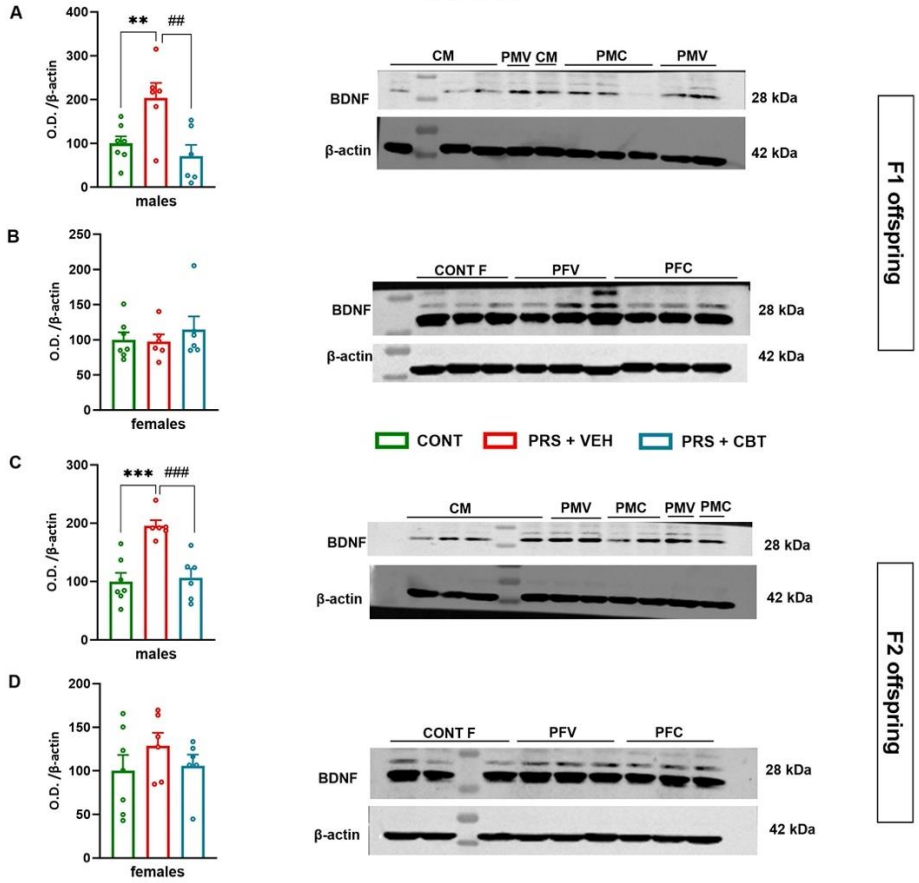
29) Turecki, G., Meaney, M.J., 2016. Effects of the Social Environment and Stress on Glucocorticoid Receptor Gene Methylation: A Systematic Review. Biol. Psychiatry 79, 87–96. https://doi.org/10.1016/j.biopsych.2014.11.022
30) van Praag, H., Kempermann, G., Gage, F.H., 2000. Neural consequences of enviromental enrichment. Nat. Rev. Neurosci. 1, 191–198. https://doi.org/10.1038/35044558
31) Verhaeghe, R., Gao, V., Morley-Fletcher, S., Bouwalerh, H., Van Camp, G., Cisani, F., Nicoletti, F., Maccari, S., 2021. Maternal stress programs a demasculinization of glutamatergic transmission in stress-related brain regions of aged rats. GeroScience 44, 1047–1069. https://doi.org/10.1007/s11357-021-00375-5
32) Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854. https://doi.org/10.1038/nn1276
33) Webster-Stratton, C.H., Reid, M.J., 2010. The incredible years program for children from infancy to pre-adolescence: Prevention and treatment of behavior problems, in: Clinical Handbook of Assessing and Treating Conduct Problems in Youth. Springer Science + Business Media, New York, NY, US, pp. 117–138. https://doi.org/10.1007/978-1-4419-6297-3_5
34) Yehuda, R., Bell, A., Bierer, L.M., Schmeidler, J., 2008. Maternal, not paternal, PTSD is related to increased risk for PTSD in offspring of Holocaust survivors. J. Psychiatr. Res. 42, 1104–1111. https://doi.org/10.1016/j.jpsychires.2008.01.002
35) Zuena, A.R., Mairesse, J., Casolini, P., Cinque, C., Alemà, G.S., Morley-Fletcher, S., Chiodi, V., Spagnoli, L.G., Gradini, R., Catalani, A., Nicoletti, F., Maccari, S., 2008. Prenatal Restraint Stress Generates Two Distinct Behavioral and Neurochemical Profiles in Male and Female Rats. PLoS ONE 3. https://doi.org/10.1371/journal.pone.0002170

S1_Supplementary Fig 1. Hippocampal BDNF and mGluR2/3 protein levels in the F1 offspring


S2_Supplementary Fig 3. Hippocampal BDNF and mGluR2/3 protein levels in the F2 offspring




S3_Supplementary Fig 5. Hippocampal BDNF protein levels in the F1 and F2 offspring following CBT treatment in F0 mothers




**S1_Supplementary Figure 1.** Representative images of immunoblots of A) BDNF and B) mGluR2/3 protein of CONT and PRS rats of F1 generation, showing results in both sexes for BDNF and only in males for mGluR2/3 (M=males, F=females). The samples framed by the black rectangle in B) were not included in the analysis because they were from an unrelated study. **S2_Supplementary Figure 2.** Representative images of immunoblots of A) BDNF and B) mGluR2/3 protein of CONT and PRS rats of F2 generation, showing results in both sexes for BDNF and only in males for mGluR2/3 (M=males, F =females). **S3_Supplementary Figure 5. PRS increased BDNF protein levels in the hippocampus of F1 and F2 male offspring.** The corrective effect of Cbt was observed up to the F2 generation with BDNF levels being restored to control unstressed levels in PRS males while no effect of PRS nor of Cbt was observed in PRS females of both generations. Representative images of the immunoblots of BDNF are shown separately for sex and generation All values are means ± S.E.M. (n=6 rats/group), **p < 0.01 and ***p < 0.001 for CONT vs. PRS, ##p < 0.01 and ###p < 0.001 for PRS+ Veh vs. PRS+ Cbt.

### B. Transgenerational transmission of gestational stress (*Article n° 2 in preparation)*

The parental environment has significant programming capacities, particularly during vulnerable periods such as gestation and the early period after the birth. Stress experienced by mothers during these critical times can have profound effects, extending beyond the directly exposed individuals to subsequent generations that have never been in the presence of the stress factor. This phenomenon, known as transgenerational inheritance of stress, has been well-documented in preclinical models mostly in rodents (Moisiadis et al., 2017; van Steenwyk et al., 2018). However, retracing this phenomenon in humans is challenging due to difficulties in conducting extensive longitudinal studies. Moreover, there is often confusion regarding the term transgenerational transmission.

In continuity with the previous section and after addressing the intergenerational transmission, this ongoing work aims to investigate whether *postpartum* treatments with carbetocin (CBT) administered to F0 dams was persistent to mitigate deficits in the F3 PRS offspring. Therefore, this ongoing work aimed to highlight whether the gestational stress experienced by F0 dams persisted in the F3 generation of males and whether CBT treatment in F0 mothers was strong enough to mitigate deficits in F3 PRS male offspring. We focused on risk-taking behavior, stress/anti-stress balance in the plasma, and assessed correlations between the F3 and their mothers (F2) and great-grandmothers (F0) to understand how far these adverse effects can extend and to determine if certain parameters in F0 dams are predictive of deficits in the offspring.We addressed also the difference between intraperitoneal (IP) and intranasal (IN) CBT administration, because IN administration of oxytocin, which is less invasive and more translationally applicable to human treatments for neuropsychiatric disorders, has demonstrated efficacy in reducing psychotic symptoms and improving social perception in schizophrenia (Pedersen et al., 2011).

By investigating these aspects, our study aims to provide insights into the potential for *postpartum* oxytocinergic activation to reverse and mitigate transgenerational deficits of PRS in offspring, using both intraperitoneal (CBT IP) and intranasal (CBT IN) treatments effects. Understanding these mechanisms is crucial for developing targeted interventions to mitigate the long-term health impacts of early-life stress across generations.

## Article n° 2 in preparation

# Gestational stress in F0 dams induces a transgenerational inheritance in the stress/anti-stress balance of male F3 offspring, while peripheral and central oxytocinergic activation via carbetocin mitigates PRS deficits up to F3

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## ABSTRACT

Environmental exposures during gestation can induce epigenetic modifications that persist across generations, impacting phenotypes in both directly exposed subjects and non-exposed subjects in subsequent generations—a phenomenon termed transgenerational inheritance. The perinatal stress rat model (which includes exposing pregnant females to gestational stress with consequent reduction in maternal behavior) induces long-lasting alterations that are transmitted intergenerationally. Here, we investigated the transgenerational effects of gestational stress on risk-taking behavior and stress/anti-stress balance in male F3 offspring, mediated through maternal behavior in F0 great-grandmothers and F2 dams. Additionally, we explored the role of maternal behavior and oxytocinergic activation *via* carbetocin (CBT) administration during the *postpartum* period in mitigating these effects, by comparing the efficacy of intraperitoneal (IP) and intranasal CBT administration (IN). We observed impaired maternal behavior and dysregulation of stress/anti-stress balance characterized by elevated corticosterone (CORT) levels and reduced oxytocin (OT) levels in F0 dams, persisting until F2 mothers. These deficits translated into reduced risk-taking in the open arms and increased exploration in the closed arms, as well as an imbalance in CORT/OT levels in F3 male offspring. Strikingly, both *postpartum* CBT IP and CBT IN treatments in F0 dams rescued these deficits, maintaining efficacy through F2 mothers and correcting PRS-induced dysregulation in F3 male offspring. Overall, our study underscores the significant role of gestational stress in the transgenerational transmission of stress-related phenotypes and emphasizes the persistence and potential of early-life pharmacological interventions, such as *postpartum* CBT, in reversing these effects. Understanding these mechanisms is crucial and can pave the way for developing targeted interventions to mitigate the long-term health impacts of early-life stress across generations.

**Keywords**: Transgenerational inheritance, transmission, gestational stress, oxytocin, HPA axis

## INTRODUCTION

When stress occurs during vulnerable periods like the perinatal stage, it can have profound effects on both individuals and their offspring, leading to metabolic and neurodevelopmental disorders (Barker, 1995; Maccari et al., 2017). The concept of transgenerational inheritance suggests that environmental exposures can induce epigenetic modifications that persist across generations that were not directly exposed, potentially influencing phenotypes in both parents and their offspring. However, the definition of transgenerational inheritance is relatively recent, and previous literature investigations misused the term transgenerational and often confused it with intergenerational (Ward et al., 2013). Indeed, environmental stressors during gestation (F0) can directly impact the fetus (F1) and its developing germ cells (F2) which refers to intergenerational transmission, when transgenerational transmission involves the transmission of deficits beyond the F2 generation to the F3 and subsequent generations (Klengel et al., 2016; Skinner, 2008; Tricker, 2015). Human studies have corroborated the inheritance concept, strikingly showing that stress and trauma can have far-reaching effects across multiple generations. Importantly, analysis of the cohort of Holocaust survivors revealed the transgenerational transmission of trauma and stress (Lev-Wiesel, 2007). and a predisposition to develop post-traumatic stress disorders (PTSD), depression, and anxiety disorders over three generations (Yehuda et al., 2008). A recent study on Leningrad siege survivors found that the effects of famine led to eating and metabolic disorders persisting across three generations (Bukatova et al., 2023). Strikingly, Champagne and collaborators reported a transgenerational continuity in cases of child abuse (Champagne, 2008). Nevertheless, the exact mechanisms by which this inheritance occurs are not fully understood. Multiple animal models corroborated the previous clinical findings and showed long-lasting phenotypic changes, affecting both behavioral and molecular dysregulations, in guinea pigs these deficits were inherited up to the 3rd generation through both parental lines (maternal and paternal line) (Moisiadis et al., 2017) and investigations in mice retraced persistence of stress impairments until the 4th (van Steenwyk et al., 2018) and even up to the 5th generation (Boscardin et al., 2022). Together, these preclinical studies contributed to understanding the mechanisms underlying transgenerational inheritance, albeit not fully elucidating them. Animal models such as the PeRinatal Stress (PRS) have been invaluable for studying stress effects (Maccari et al., 1995), studies using the model highlighted that gestational stress decreased maternal care during the first *postpartum* week (Gatta et al., 2018) and increased maternal glucocorticoids, disrupting the hypothalamic-pituitary-adrenal (HPA) axis activity and feedback regulation in the offspring (Maccari et al., 1995). Furthermore, PRS deficits have been observed to persist in adults and aged PRS males (Gatta et al., 2018; Verhaeghe et al., 2021). We have recently shown that PRS-related deficits can occur in the stress-exposed F0 dams and their F1 offspring retracing deficits in corticosterone (CORT) and oxytocin (OT) levels up to F2 generations of PRS males and females which are associated to impaired maternal behavior in F0 and F1 mothers. Additionally, F1 and F2 PRS offspring showed

altered gene expression of glucocorticoid (GR), mineralocorticoid receptors (MR), and BDNF (Brain-derived neurotrophic factor), along with promoter methylation imbalances of the previous genes (work submitted). Moreover, the correction of maternal behavior with *postpartum* CBT in F0 showed the persistence of the increase of maternal behavior until F1 rat dams, (work submitted), thus underscoring the pivotal role of maternal behavior and OT in the intergenerational programming induced by maternal stress. It is crucial to understand how stress-related disorders develop and persist across generations, revealing underlying biological mechanisms. Moreover, the findings can inform interventions to break the cycle of stress transmission, thereby improving mental and physical health outcomes across generations.

For this reason, here we aimed to investigate the transgenerational impact of stress through the maternal line in the PRS model at both behavioral and endocrine levels. We also focused on the pivotal role of maternal behavior in buffering these deficits using intraperitoneal or intranasal carbetocin (CBT IP or CBT IN) treatments administered during the first week of the *postpartum* period. Understanding the mechanisms and potential interventions for transgenerational stress transmission can address the long-term health implications for affected populations.

## MATERIAL AND METHODS

### Animals

All experiments followed the rules of Directive 2019/10/10 of the Council of the European Communities and the Comité d'Ethique CEEA-75 (Comité d'Ethique en Expérimentation Animale Nord-Pas de Calais). This project was approved by the MESRI (Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation ; authorization #33654).

20 Nulliparous Sprague Dawley female rats (Charles River, France) weighing approximately 250 g, were purchased from Charles River (France) and housed under standard conditions with a 12 h light/dark cycle (lights on 7 am: lights off 7 pm), with *ad libitum* access to food (SAFE A04-10, France, 72.4% carbohydrates, 19.3% proteins, 8.4% lipids) and water. Upon arrival in the animal facility, the animals were placed in large cages (between 3 to 5 animals per cage) for 10 days to acclimatize and synchronize their estrous cycle. After group housing (five females/cage) for two weeks, each female was individually housed for one week with a sexually experienced male rat. Following that, a gain of at least 10 grams was considered as an index of pregnant status. On Embryonic day 11 (E11), pregnant females were then randomly assigned to either the stress (n= 13) or the control (n= 7) group. For the rest of the procedure, all animals including the generated offspring after mating were housed under standard conditions as previously mentioned, all procedures and experimentations were in adult male and female rats (2.5 to 3 months old).

**Maternal study**

1. **Maternal line procedure and experimental design**

This paper investigates the transgenerational transmission of stress from F0 to F3 generations, the experimental design is described in **Figure 1A**. Adult F0 females from each group were paired with control naive males to produce F1 offspring. To obtain subsequent generations, females of groups of interest were mated with control males, this process was repeated to generate F2 and F3 generations. Gestational stress was induced in F0 dams and carbetocin (CBT) treatments were administered solely to F0 dams from *postpartum* day 1 to *postpartum* day 7. Four groups were generated from the F0 dams: control undisturbed dams (CONT), stressed dams (Stress treated with vehicle), stress-treated dams with intraperitoneal CBT (Stress CBT IP), and stress-treated dams with intranasal CBT (Stress CBT IN). Subsequent generations of mothers (F2) and offspring (F3) were produced from the F0 dams, resulting in four experimental groups: control (CONT), descendants of stressed F0 dams (PRS), descendants of stress-treated dams with intraperitoneal CBT (PRS CBT IP), and descendants of stress-treated dams with intranasal CBT (PRS CBT IN). No control group with CBT was included in this study since previous reports didn't show any effect of CBT in the control (Gatta et al., 2018, Morley-Fletcher et al., 2024).

To better highlight the transgenerational reprogramming, first F0 dams represent great-grandmothers (F0 dams or mothers= F0 great-grandmothers) for F3 offspring while F2 dams represent their mothers (F2 dams= F2 mothers), finally F0 dams are the grandmothers of F2 dams. Overall, each generation was identified as F0, F1, and F2 for mothers and their corresponding offspring in F1, F2, and F3, creating a genealogical table showing the relationship between mothers x offspring and offspring x great-grandmothers.

2. **Maternal care analysis**

Maternal care (behavior) was monitored for 24 h every day during the first week of lactation (PP1-PP7). Constant monitoring was performed with small infrared cameras placed on the animal cage rack where cages containing lactating females were placed. Continuous recording was conducted around, 24 hours a day, with the digital video signals being transmitted to a computer for storage on a dedicated hard disk. The Video Viewer Application® (version 0.1.8.4) served as the software tool for controlling video recording and playback. Within each observation period, the behavior of each mother was scored every minute from PP1 to PP7 (60 observations/h with 1 h of observation per day, from 7 am to 8 am. The active behavior of the mother in the nest (nursing behavior, licking, carrying pups, and arched back over pups) was scored, and the data obtained were expressed as a percentage of maternal care with respect to the total number of observations then the mean over the week for each

group was reported, thus allowing for a comprehensive understanding of maternal response to the gestational stress (Gatta et al., 2018).

### 3. Maternal response to pups' separation test

In addition to the observation of natural home-cage behavior, we investigated maternal reactivity following a brief separation from pups. On the seventh day after giving birth (PP7), at approximately 1 pm, we temporarily separated mother rats from their pups for 15 minutes. During this separation, we recorded the weights of both the mothers and the pups, and the pups were sex-identified. Before reuniting them, we dispatched the pups. Once reunited, we measured the latency to initiate contact with her pups, each point in the graph representing a different mother and its associated latency for dams-pups contact.

### 4. Stress procedure imposed in F0 mothers

Gestating females (n= 13) in the stress group were subjected to a restraint stress procedure according to the standard protocol of Maccari's group (Gatta et al., 2018; Morley-Fletcher et al., 2024). From day 11 of pregnancy until delivery, the dams were subjected to three stress sessions per day (45 min each during the light phase around 9:00 am, 12:00 pm, and 3:00 pm.), during which they were taken to a different room (Pre-room) and placed in transparent plexiglass cylinders with conical end caps and exposed to bright light (979 luxe). Each stress session was separated at least by 2 hours of intervals. The control dams (n= 7) were left undisturbed. At birth, pups were left undisturbed with their mother until weaning 21 days after birth. Both male and female rats were used in the present study. The local ethical committee approved the gestational restraint procedure.

### 5. Oxytocinergic activation *via postpartum* carbetocin treatments

In lactating dams, post-partum (day until day 7) treatment was imposed only on F0 mothers using either vehicle (saline) or carbetocin (CBT; 1 mg/kg, SP080756, Polypeptide group, Strasbourg, France) *via* intraperitoneal (IP) or intranasal (IN) administration. The dose and route of administration of carbetocin IP were selected based on previous reports (Gatta et al., 2018; Morley-Fletcher et al., 2024), and for IN the intervention was adapted from oxytocin nasal administration used by Calcagnoli and his collaborators; the intervention lasted 30 seconds per rat maximum, the conscious rat was held by the experimenter in a supine position with a horizontal head position. The solution (2 × 10 ul) was bilaterally applied using a 100 ul pipette and equally distributed on the squamous epithelium of both the left and right rhinarium, avoiding direct contact of the tip of the pipette with the rhinarium, or direct application into one of the nostrils or in proximity of the philtrum (Calcagnoli et al., 2015). After all treatments, the rats were returned to their home cage.

### 6. Maternal stress/anti-stress balance measurements

After weaning around pp30, brains were rapidly removed and the trunk blood was collected. Blood and tissue collection occurred between 9 am and 11h30 am to avoid hormonal variation that occurs during the day. endocrine parameters (OT and CORT) were determined in plasma extracted from trunk blood samples. Plasma was collected using 20ul ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and 4ul protease inhibitors and centrifuged for 15 min at 1000 g at 4°C to isolate the plasma, then it was stored at -20°C until assessment. OT (sensitivity 9.4 pg/ml; CUSABIO # CSB-E14197r) and CORT (sensitivity 6.1 ng/mL; Demeditec # DEV9922) were determined in plasma using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol. All standards, samples, and controls were analyzed concurrently in duplicate. OT and CORT levels were calculated using a calibration curve generated by kit standards and analyzed on the website MYASSAY.COM.

**Progeny study**

1. **Risk-taking behavior and exploratory behavior in the elevated plus maze**

At 3 months of age, male adult offspring from control, PRS/stress, or PRS/stress treated mothers (n= 13-20 animals/group) were assessed in the elevated plus maze (EPM) between 9:30 am to 11:30 am, the luminosity parameters of the maze are the following; in the center= 25 lux, in the Open arms= 35 lux, Closed arms= 14 lux. The test was performed for 5 min and began with the placement of the rat in the center of the maze with the head facing a closed arm. The apparatus used consists of four arms (two open without walls and two closed by 30 cm high walls), each 50 cm long and 10 cm wide. The different parameters were recorded with a video camera tracking using specific software (EthoVision, Noldus, EthoVision, The Netherlands). For risk-taking behavior, the following parameters were represented; the % of time spent in the open arms, latency, and frequency of visits in the open arms. For exploratory behaviors in the EPM, time spent in the closed arms, latency, and frequency of visits in the closed arms were calculated and represented.

2. **Stress/anti-stress balance after novelty stress test**

At least one week after EPM assessment, reactivity of the HPA axis in response to stress was analyzed in adult control and PRS progeny (n = 6-10 per group) of dams treated with either saline or carbetocin during the *postpartum* period. The animals were submitted to a 30-minute novelty stress exposure during the first half of the light phase of the light/dark cycle (between 9:00 a.m. and 12:00 p.m.).

Novelty stress consisted of placing animals in a transparent cylindrical Plexiglas cage (30 cm diameter, 50 cm high) without sawdust and under a bright light (400 lx). Blood samples from the tail vein were collected before stress (T0) and 30 min afterward. Plasma was isolated using an anticoagulant (20 µl ethylenediaminetetraacetic acid; EDTA) and protease inhibitors (4 µl ), then centrifuged for 15 min at 1000 x g at 4°C to isolate the plasma, then it was stored at -20°C until

assessment. OT (sensitivity 9.4 pg/ml; CUSABIO # CSB-E14197r) and CORT (sensitivity 6.1 ng/mL; Demeditec # DEV9922) were determined in plasma using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol. All standards, samples, and controls were analyzed concurrently in duplicate. OT and CORT levels were calculated using a calibration curve generated by kit standards and analyzed on the website MYASSAY.COM.

**Statistical analysis**

All graph representations were done on GraphPad PRISM, and statistical analyses were performed on STATISTICA 8.0 (Stat Soft. Inc). The normality of data distribution was assessed using the Shapiro-Wilk test, after that a Two-ways ANOVA followed with Newman-Keuls post-hoc (NK) or Planned Comparisons (PC) tests were assessed. Pearson correlations analysis were used to investigate association between F3 offspring and their F2 mothers and then with their F0 great-grandmothers. A p-value $< 0.05$ was considered as statistically significant. Independent variables included groups (*: PRS *vs*. CONT) and treatments (#: PRS *vs*. PRS + CBT IP) and ($: PRS *vs*. PRS + CBT IN). A p-value of $< 0.05$ was considered statistically significant. Graphical representations were created using GraphPad Prism version 10.2.3.

**RESULTS**

The paradigm used in this study is illustrated in **Figure 1A**. The approach adopted here examines the potential transgenerational transmission of stress and the corrective effect of CBT administered during the post-partum period solely in F0 mothers. This stress model, as outlined by Maccari in 1995 (Maccari et al., 1995), involves restraint stress. The CBT treatment was administered after delivery, spanning from *postpartum* day 1 (PP1) to *postpartum* day 7 (PP7). Subsequently, behavioral and molecular analyses were conducted on the mothers and the offspring. This study design allowed for the investigation to highlight the importance of the *postpartum* period and the transmission of stress.

**Gestational stress in F0 mothers impacted maternal behavior and maternal reactivity until the F2 generation**

Gestational stress in F0 dams had direct impact on F0 lactation dams (grey inset; Maternal care; $n^{(7, 4, 6, 3)}$, ($F_{(3,16)}$=10.95, p=0.0003; $PC^{(Cont\ vs\ STRESS)}$ p= 0.00009, $^{(STRESS\ vs\ STRESSCBT\ IP)}$ p= 0.0001, $^{(STRESS\ vs\ STRESS\ CBT\ IN)}$ p= 0.0005; Maternal reactivity; $n^{(7, 6, 4, 3)}$, ($F_{(3,16)}$=4.07, p=0.02; $PC^{(Cont\ vs\ STRESS)}$ p=0.05, $^{(STRESS\ vs\ STRESSCBT\ IP)}$p=0.01, $^{(STRESS\ vs\ STRESS\ CBT\ IN)}$ p=0.007), furthermore the deficit persisted and impacted F2 dams by reducing licking/nursing behavior **(Figure 1B)**, and it also reduces maternal reactivity by increasing the latency to make the first contact with pups after separation (F2 maternal care, $n^{(Cont)}$= 8, $n^{(PRS)}$= 8, $n^{(PRS\ CBT\ IP)}$= 7, $n^{(PRS\ CBT\ IN)}$= 5; group effect, $F_{(3, 24)}$=3.71; p= 0.025; $PC^{(Cont\ vs\ PRS)}$ p= 0.01; $^{(PRS\ vs\ PRS\ CBT\ IP)}$ p= 0.01; $^{(PRS\ vs\ PRS\ CBT\ IN)}$ p= 0.01) (F2 maternal reactivity, $n^{(8, 8, 7, 5)}$; group effect, $F_{(3, 24)}$=5.60;

p= 0.004; PC$^{(\text{Cont vs PRS})}$ p= 0.0006; $^{(\text{PRS vs PRS CBT IP})}$ p= 0.01; $^{(\text{PRS vs PRS CBT IN})}$ p= 0.01) **(Figure 1C)**. Analysis between maternal care and maternal reactivity showed a negative correlation in both F2 and F0 dams (F2 r= -0.38; p= 0.04, F0 r= -0.40; p= 0.04). Moreover, for maternal care, an evident correlation occurred among F0 mothers (Great-grandmother) and F2 mothers, with a significant positive correlation (r= 0.42; p= 0.03), which shows that deficits that occur in F0 can be predictive of deficits in F2 mothers **(Figure 1D)**.

**Stress/anti-stress balance is impacted by stress**

When looking into endocrine parameters, PRS deficits did not persist in F2 mothers on the CORT levels ($n^{(7,\ 8,\ 6,5)}$, $F_{(3,22)}$= 1.54, p= 0.23), but deficits in the F0 dams were evident with an increase of CORT levels in stressed dams that was corrected in mothers that received CBT IP and CBT IN during *postpartum* (grey inset; $n^{(5,\ 5,\ 5,3)}$, ($F_{(3,14)}$= 6.35, p= 0.006; PC$^{(\text{Cont vs STRESS})}$ p= 0.001, $^{(\text{STRESS vs STRESSCBT IP})}$ p= 0.007, $^{(\text{STRESS vs STRESS CBT IN})}$ p=0.007) **(Figure 1E)**. Interestingly, OT levels that were reduced in F0 dams (grey inset; $n^{(5,\ 5,\ 5,3)}$, ($F_{(3,14)}$= 3.11, p= 0.06; PC$^{(\text{Cont vs STRESS})}$ p= 0.04, $^{(\text{STRESS vs STRESSCBT IP})}$ p= 0.03, $^{(\text{STRESS vs STRESS CBT IN})}$ p=0.10) and that was retraced in the plasma of F2 mothers, and both CBT IP and CBT IN mitigated the impairments (n= 5, 4, 6, 3, $F_{(3,14)}$= 3.41, p= 0.04), PC$^{(\text{Cont vs PRS})}$ p= 0.04, $^{(\text{PRS vs PRS CBT IP})}$ p= 0.05, $^{(\text{PRS vs PRS CBT IN})}$ p= 0.05) **(Figure 1F)**.

**Transgenerational transmission of risk-taking behavior deficits in the PRS in male offspring**

When investigating the programming effects at the behavioral level on the F3 male offspring, it was found that stress experienced by F0 mothers significantly reduced risk-taking and exploratory behaviors in F3 PRS males. This reduction was corrected by *postpartum* CBT administered to their great-grandmothers via IP and IN routes. Specifically, analysis of risk-taking behavior in the EPM revealed a substantial decrease in the percentage of time spent in the open arms and the frequency of visits to the open arms, accompanied by increased latency to the first visit. These parameters were significantly improved in the treatment groups, indicating that CBT effectively mitigated the transgenerational effects of stress on these behaviors ($n^{(18,\ 13,\ 19,\ 20)}$; *%Open arms*; $F_{(3,\ 66)}$= 5.27, p= 0.002; PC$^{(\text{CONT vs PRS})}$p= 0.009; $^{(\text{PRS vs PRS CBT IP})}$p= 0.009; $^{(\text{PRS vs PRS CBT IN})}$p= 0.0001; *Latency to open arms*; $F_{(3,\ 66)}$= 3.34, p= 0.02; PC$^{(\text{CONT vs PRS})}$p= 0.01; $^{(\text{PRS vs PRS CBT IP})}$p= 0.005; $^{(\text{PRS vs PRS CBT IN})}$p= 0.01; *Frequency of visits in the open arms*; $F_{(3,\ 66)}$= 3.86, p= 0.01; PC$^{(\text{CONT vs PRS})}$p= 0.04; $^{(\text{PRS vs PRS CBT IP})}$p= 0.03; $^{(\text{PRS vs PRS CBT IN})}$p=0.001) **(Figure 2A)**.

Exploratory behavior in the EPM appeared to be less impacted overall. PRS males exhibited an increase in the time spent in the closed arms. This increase was reduced in the groups treated with CBT. However, other parameters, such as the latency to enter the open arms and the frequency of visits to the open arms, were not significantly affected by either stress or CBT treatment ($n^{(18,\ 13,\ 19,\ 20)}$;

Time in the closed arms; $F_{(3, 66)}$= 8.22; p= 0.0001; $PC^{(CONT\ vs\ PRS)}$p= 0.004; $^{(PRS\ vs\ PRS\ CBT\ IP)}$p= 0.001; $^{(PRS\ vs\ PRS\ CBT\ IN)}$p= 0.000005) **(Figure 2B)**.

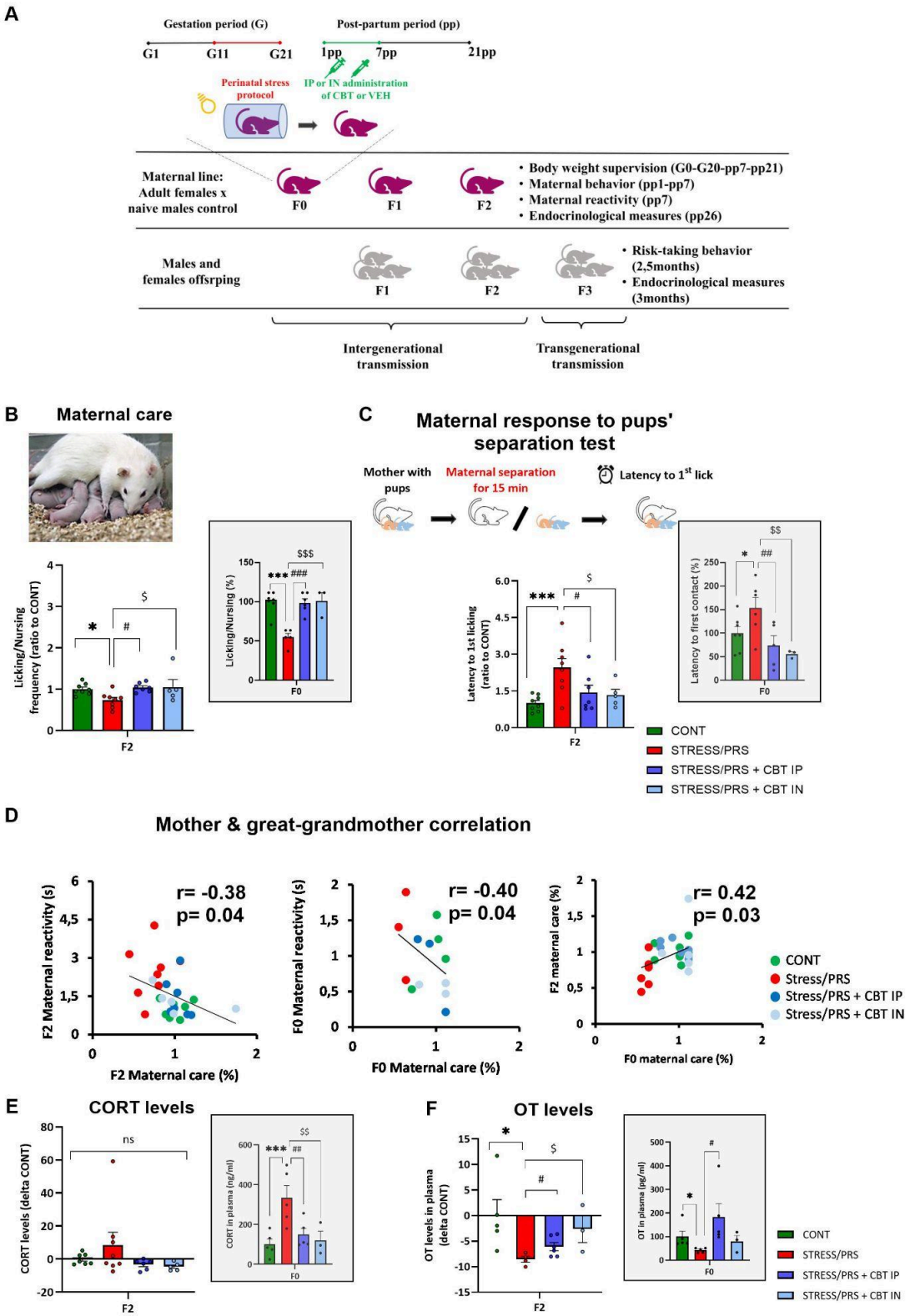

**Figure 1. Deficits due to F0 gestational stress persist until F2 rat mothers in an intergenerational manner.** Experimental timeline **(A)**. Maternal care in F2 dams and F0 in inset (grey), stress reduced maternal care in both generations and CBT treatment (IP and IN) restored it **(B)**. F2 and F0 (inset grey) maternal response to pups' separation on PP7. Gestational stress increased latency to lick and that was corrected by both CBT IP and CBT IN **(C )**. Pearson's correlation between F0 and F2 maternal behavior, coefficient (r) values, and related p values **(D)**. Stress/anti-stress balance, CORT levels were increased by stress in F0 dams and corrected by the *postpartum* treatment (grey inset), but F2 CORT was not impacted when OT levels were impaired in both F0 (grey inset, OT represented in % of values and CONT are put to 100%) and F2 dams, and the treatment successfully increased OT levels, F2 results were represented as delta to CONT dams **(E-F)**. All values are means ± S.E.M. (n= 3-8 rats/group), * $p < 0.05$ and ***$p < 0.001$ for CONT *vs*. STRESS or PRS; and # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for STRESS or PRS *vs*. STRESS or PRS + CBT IP, and $ $p < 0.05$, $$ $p < 0.01$ and $$$ $p < 0.001$ for STRESS or PRS *vs*. STRESS or PRS + CBT IN.

**PRS males exhibited a disturbed Stress/anti-stress balance in a transgenerational manner**

After exposure to novelty stress, hormone concentrations were assessed in the plasma of F3 PRS males, it scored higher levels of CORT that were significantly reduced in the treated groups (CORT; $n^{(7,\ 10,\ 8,\ 6)}$; $F_{(3,\ 27)}= 5.08$, p= 0.006; $PC^{(CONT\ vs\ PRS)}$p= 0.03; $^{(PRS\ vs\ PRS\ CBT\ IP)}$p= 0.02; $^{(PRS\ vs\ PRS\ CBT\ IN)}$p= 0.0008) **(Figure 2C)**. On the other hand, the neuropeptide OT was strikingly reduced in the PRS males, on the other hand in the CBT IP treated group the levels were normalized to the control, but no significant differences were observed in the CBT IN groups ($n^{(\ 9,\ 10,\ 6,\ 6)}$; $F_{(3,27)}= 2.44$, p= 0.08; $PC^{(CONT\ vs\ PRS)}$p= 0.02; $^{(PRS\ vs\ PRS\ CBT\ IP)}$p= 0.03; $^{(PRS\ vs\ PRS\ CBT\ IN)}$p= 0.14) **(Figure 2D)**. Overall, in F3 adult males, Pearson's correlation showed a significant positive association between OT levels and their risk-taking behavior (r= 0.37; p= 0.05) **(Figure 2E)**, with no significant association made with CORT **(Supplementary Table 1)**. Furthermore, risk-taking behavior in F3 males correlated strongly with their F2 mothers' characteristics, including maternal care (r= 0.37; p= 0.05), maternal reactivity (r = -0.39; p = 0.002), and maternal corticosterone (CORT) levels (r= 0.44; p= 0.01). These correlations suggest that the maternal behaviors and stress responses of F2 mothers significantly influence the risk-taking behaviors observed in their F3 offspring **(Figure 2F)**. Further correlations are presented in **(Supplementary Table 1)**.

**F0 great-grandmothers and F2 mothers programs risk-taking behavior in F3 male offspring**

Surprisingly, F0 mothers exhibit predictive power regarding risk-taking behavior in a transgenerational manner, extending to F3 male offspring **(Figure 2E)**.

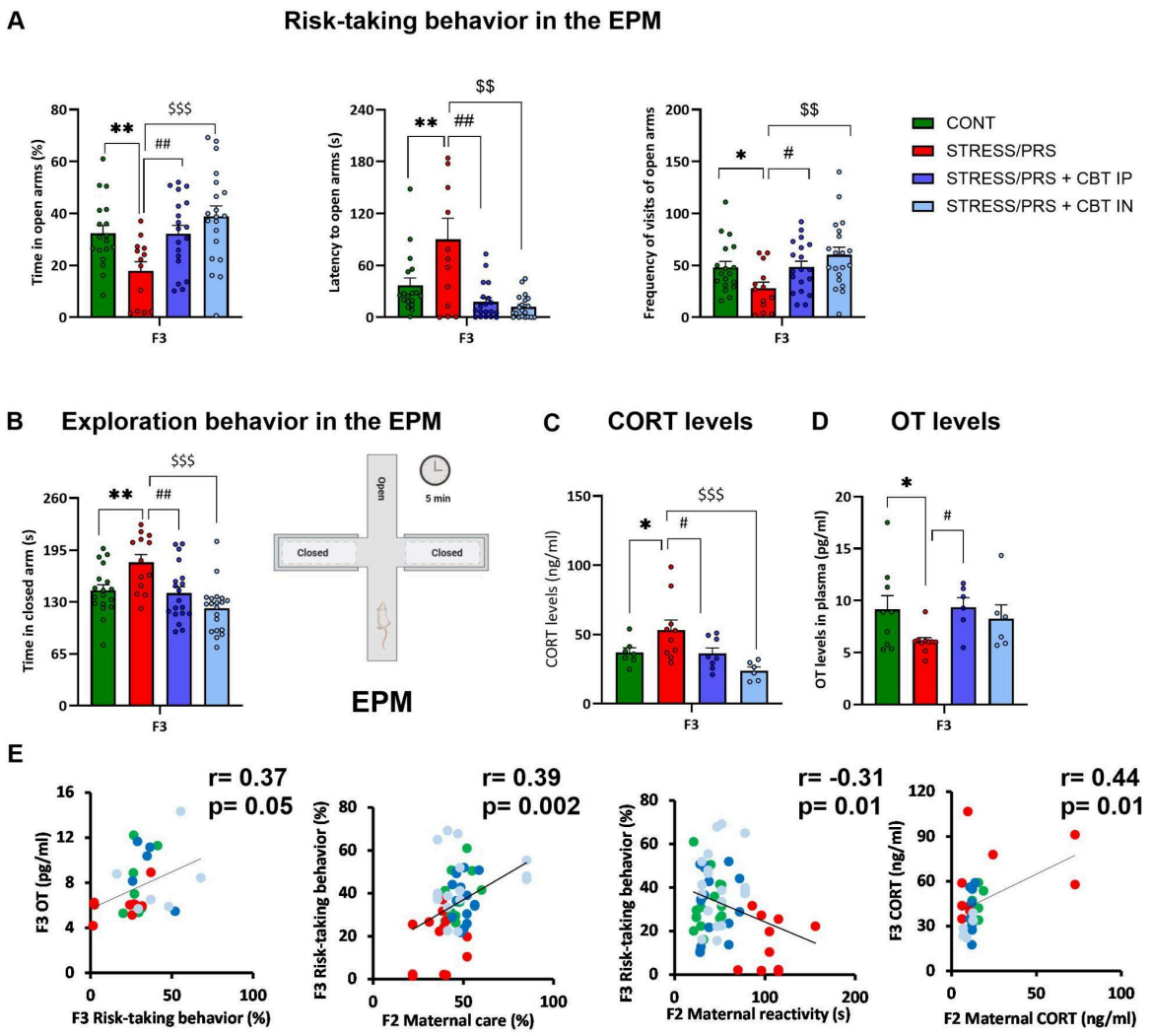


**Figure 2. Transgenerational transmission of PRS deficits in adult F3 offspring.** Risk-taking behavior was impaired in PRS males when the offspring from CBT groups showed a corrected risk-taking behavior in the time spent in open arms, latency, and frequency of visits in the open arms **(A)**. Exploration in the EPM was reduced in PRS males in the time spent in closed arms when there was no significant impact on the latency and frequency of visits in the closed arms **(B)**. Stress/anti-stress balance was impaired in F3 PRS males and mitigated in the CBT IP and CBT IN groups **(C-D)**. OT correlation with risk-taking behavior among the F3 offspring **(E)**. Risk-taking behavior correlation with F2 mothers **(F)**. All values are means ± S.E.M. (n=3-20 rats/group), * $p < 0.05$ and **$p < 0.01$ for CONT *vs*. STRESS or PRS; and # $p < 0.05$ and ## $p < 0.01$ for STRESS or PRS *vs*. STRESS or PRS + CBT IP, and \$ $p < 0.05$, \$\$ $p < 0.01$ and \$\$\$ $p < 0.001$ for STRESS or PRS *vs*. STRESS or PRS + CBT IN.

### F0 maternal behavior predicts stress and anti-stress balance in F3 offspring

Notably, a robust positive correlation is observed between risk-taking behavior in F3 males and F0 maternal care (r= 0.48; p= 0.0001), while negative correlations were evident with F0 maternal reactivity to pups separation (r= -0.48; p= 0.0001) and F0 maternal CORT (r= -0.55; p= 0.0001) as illustrated in **(Figure 3A)**, indicating the influence of transgenerational programming and blueprinting. Nevertheless, maternal behaviors in F0 great-grandmothers show a high correlation with

the stress hormone balance of F3 offspring, showing a strong negative correlation between maternal care and F3 CORT level (r= -0.55; p= 0.001), and a positive one between maternal reactivity and F3 CORT (r= 0.58; p= 0.0007). Additionally, the anti-stress hormone OT in F3 was positively correlated with maternal care (r= 0.39; p= 0.04) and a tendency for correlation with maternal OT levels (r= 0.35; p= *0.06*) in F0 great-grandmothers **(Figure 3B)**.

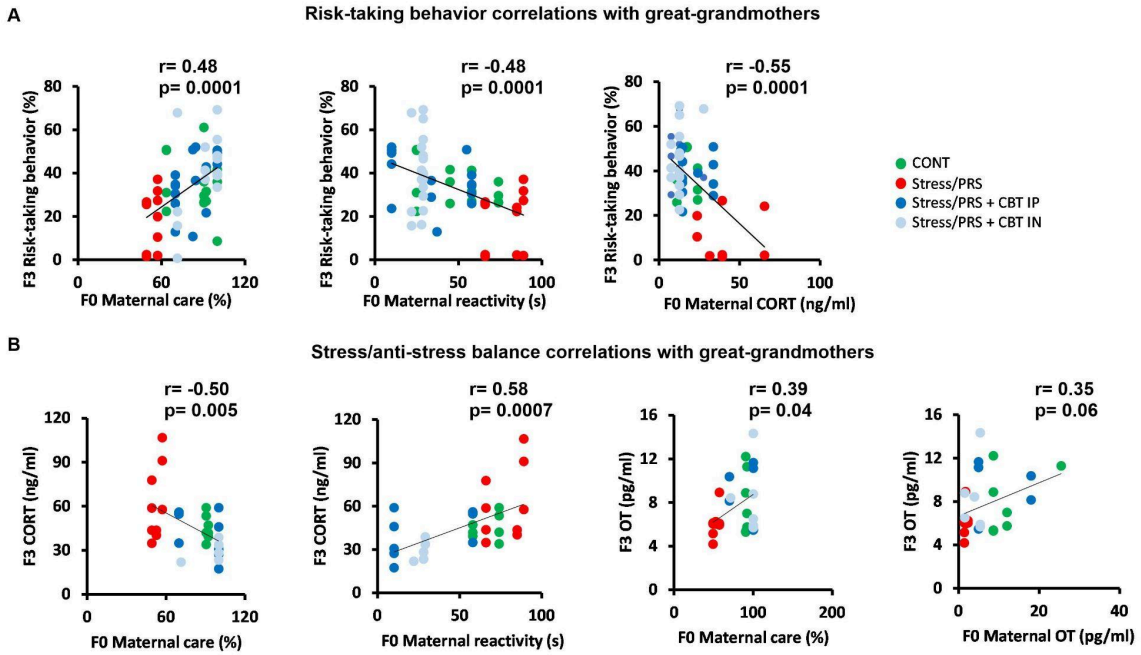


**Figure 3. Transgenerational transmission of PRS deficits correlated with F0 great-grandmothers.** Correlation analysis of F3 offspring parameters **(A)** Risk-taking behavior and **(B)** stress/anti-stress balance) with F0 great-grandmothers parameters (Maternal behaviors and stress/anti-stress balance) Pearson's correlation coefficient (r) values and related p values are reported in Table 1 (n= 7-20 rats /group).

## DISCUSSION

The present study used the maternal line in the PRS rat model to investigate the transgenerational transmission of stress, and the potential effects of Carbetocin (CBT) administered exclusively to F0 mothers during the *postpartum* period. This investigation is the first of its kind to show transgenerational transmission of maternal stress accompanied by a transgenerational correction of its deficits through the increase of maternal behavior. Previous studies on transgenerational inheritance focused more on highlighting the inheritance but not on pharmacological approaches to correct it. Indeed, here we showed an impaired maternal behavior in F0 dams that persisted until F2 dams inducing deficits evident in PRS F3 offspring which was underscored by a dysregulation of the stress/anti-stress balance through an increase of CORT levels and a reduction of OT levels in the plasma after 30 minutes exposure to novelty stress, these endocrine deficits were accompanied by a reduced risk-taking behavior and exploration behavior in the EPM in F3 PRS adult males. Strikingly,

both *postpartum* CBT IP and CBT IN treatments in F0 dams, rescued all the deficits, and the treatment was sufficient to mitigate deficits until F2 mothers and even succeeded in correcting PRS-related dysregulation in F3 male offspring.

Gestational stress in F0 mothers significantly impacted maternal behavior and reactivity in F2 dams, evidenced by reduced licking/nursing behavior and increased latency to pup contact. This suggests an intergenerational transmission of maternal care deficits, with implications for offspring development, corroborating previous findings on the intergenerational effects of ELS on maternal care (Champagne and Meaney, 2006; Meaney, 2001). Moreover, the correlation between maternal care in F0 and F2 generations supports the hypothesis that maternal behaviors can be predictive of similar behaviors in subsequent generations. Such effects may be mediated by epigenetic modifications, including changes in DNA methylation and histone acetylation, which are known to influence gene expression, especially genes related to stress response and maternal behavior (Champagne, 2008). A recent study in mother-infant dyadic interactions from Central Africa revealed that mothers' traumatic narrations led to reduced visual engagement and increased infant self-stimulation, thus highlighting the importance of maternal presence in infant coping strategies (Dozio et al., 2020).

Furthermore, our results in stressed mothers showed a *postweaning* reduction in OT levels in the plasma of F0 dams which persisted in F2 dams, and both routes of CBT treatment increased the OT levels, reflecting the increase of maternal behavior through CBT. Surprisingly CBT IP, which was reported to not cross the Blood-Brain-Barrier (BBB) (Dvorská et al., 1992; Ermisch et al., 1985), succeeded in increasing OT levels in the plasma, which may be explained by the fact that stress exposure and hormonal imbalance can increase the permeability of the BBB, indeed restraint stress-induced hyperpermeability and damage of the BBB in adult rats (Xu et al., 2019). Additionally, CBT through the feed-forward regulation, boosts OT production in the hypothalamus. This mechanism presents a promising therapeutic opportunity for addressing early-life stress mitigation (Kenkel et al., 2019). On the other hand, IN route has already been reported to directly access the brain through the olfactory nerves and tegmental nerves (Yao and Kendrick, 2022), as IN administration of OT can be absorbed and detected in the brain (Lee et al., 2020), and exert effects on the HPA axis activity, attenuating the ACTH in non-human primates blood (Parker et al., 2005). Thus, corroborating our result of CORT reduction in PRS CBT-treated animals. However it is still not clear if OT acts directly at the brain and/or adrenal level, but some recent investigations showed that OT IN produces its effects on the brain by increasing peripheral blood concentrations (Yao et al., 2023); which explains its efficiency in the two types of treatments on mitigating stress and PRS deficits in the rat mothers and great-grandmothers. In the present study IP and IN administrations did not show any significant difference when compared together, however the literature compares the efficacy and pharmacokinetics of OT when using both routes. In rodent models, IP doses typically range from 0.1

to 10 mg/kg, requiring higher doses due to systemic distribution and metabolism (Neumann et al., 2013; Takayanagi and Onaka, 2022; Yao and Kendrick, 2022). In contrast, IN administration achieves similar therapeutic outcomes with lower doses (20 to 50 µg per animal) by directly delivering OT to the brain, bypassing the blood-brain barrier (Hanson and Frey, 2008). Comparative studies suggest that while both routes mitigate stress-related behaviors, IN administration offers more rapid and targeted effects with reduced peripheral side effects (Calcagnoli et al., 2015). Additionally, a growing body of research supports the use of intranasal OT administration in clinical cases. For example, insecure individuals show improved emotional, behavioral, and neural responses to infant crying after intranasal OT treatment (Riem et al., 2016). Furthermore, this route of administration has been shown to reduce amygdala activity when listening to an infant's crying (Riem et al., 2011).

Even if maternal CORT impact was more pronounced in F0 stressed dams, than in F2 PRS dams where it showed no significant impact and a discontinuity in stress programming when it came to these last endocrine parameters. A transgenerational study in guinea pigs treated with synthetic glucocorticoids showed this kind of discontinuity and loss of epigenetic memory in some of the stress generations but that did not prevent the transmission of stress to subsequent generations showing that the deficits transmission chain was not completely broken, indeed in the maternal line males showed no significant impact of HPA axis (cortisol levels) in F2 while evident impairment in the F3 generation reappeared (Moisiadis et al., 2017). These phenomena were also observed in clinical studies, in a Ten-Year Follow-Up Study of PTSD Diagnosis in Aging Holocaust Survivors, results showed that PTSD symptom severity generally diminishes over time, however in a small subsample, there is a delayed onset, and sometimes the symptoms tended to show a worsening (Yehuda et al., 2009).

In this study, the PRS model, using gestational stress, emerges as a significant contributor to our understanding of transgenerational epigenetic inheritance. This model is notable for its ability to demonstrate transmission effects spanning up to the 3rd generation, a depth of inheritance previously documented in rodents (Moisiadis et al., 2017; van Steenwyk et al., 2018). The PRS model showed a strong transgenerational programming capacity in F3 males by reducing risk-taking behavior and impairing the stress/anti-stress balance, consistent with findings in adult and aged offspring of the PRS model (Gatta et al., 2018; Marrocco et al., 2012).

The correction of risk-taking behavior deficits by CBT IP and CBT IN administrations highlighted the anti-stress capacity of OT, as evidenced by the direct correlation between F3 OT levels and risk-taking behavior. Interestingly, F3 results in the EPM correlated with maternal care and maternal CORT levels in F2 mothers, with a stronger correlation observed with F0 dams' maternal care and stress/anti-stress balance, underscoring the enduring impact of maternal experiences on offspring outcomes. These findings align with the concept of "biological embedding" (Hertzman, 1999), where early-life

experiences shape developmental trajectories and influence later-life outcomes, including risk-taking behavior (Champagne and Curley, 2009; Curley et al., 2011). Furthermore, the stress/anti-stress balance was transgenerationally impaired, as F3 PRS males exhibited heightened CORT levels following exposure to novelty stress. However, this hyperresponsiveness was attenuated in PRS males descending from F0-treated dams with CBT, suggesting a potential therapeutic benefit. Additionally, OT levels were reduced in PRS males but normalized in the CBT IP group, indicating a restoration of oxytocinergic balance with CBT intervention. These findings are consistent with the established role of OT as an anti-stress regulator (for central OT actions) and its involvement in the HPA axis activity (Dief et al., 2018).

Finally, our investigation provides compelling evidence for the transgenerational transmission of stress and underscores the potential of carbetocin as a therapeutic intervention to mitigate these effects. The findings highlight the critical role of maternal behavior and endocrine regulation, suggesting that targeting the oxytocinergic activation solely in the first exposed generation during the *postpartum* period is sufficient to break the cycle of stress transmission.

**REFERENCES**


1) Barker, 1995. Fetal origins of coronary heart disease. BMJ 311, 171–174.
2) Boscardin, C., Manuella, F., Mansuy, I.M., 2022. Paternal transmission of behavioural and metabolic traits induced by postnatal stress to the 5th generation in mice. Environ. Epigenetics 8, dvac024. https://doi.org/10.1093/eep/dvac024
3) Bukatova, S., Reichova, A., Bacova, Z., Bakos, J., 2023. Neonatal oxytocin treatment alters levels of precursor and mature BDNF forms and modifies the expression of neuronal markers in the male rat hippocampus. Neuropeptides 102, 102384. https://doi.org/10.1016/j.npep.2023.102384
4) Calcagnoli, F., Kreutzmann, J.C., de Boer, S.F., Althaus, M., Koolhaas, J.M., 2015. Acute and repeated intranasal oxytocin administration exerts anti-aggressive and pro-affiliative effects in male rats. Psychoneuroendocrinology, This issue includes a Special Section on Biomarkers in the Military - New Findings from Prospective Studies 51, 112–121. https://doi.org/10.1016/j.psyneuen.2014.09.019
5) Champagne, F.A., 2008. Epigenetic Mechanisms and the Transgenerational Effects of Maternal Care. Front. Neuroendocrinol. 29, 386–397. https://doi.org/10.1016/j.yfrne.2008.03.003
6) Champagne, F.A., Curley, J.P., 2009. Epigenetic mechanisms mediating the long-term effects of maternal care on development. Neurosci. Biobehav. Rev. 33, 593–600. https://doi.org/10.1016/j.neubiorev.2007.10.009
7) Champagne, F.A., Meaney, M.J., 2006. Stress during gestation alters postpartum maternal

care and the development of the offspring in a rodent model. Biol. Psychiatry 59, 1227–1235. https://doi.org/10.1016/j.biopsych.2005.10.016

8) Curley, J., Jensen, C., Mashoodh, R., Champagne, F., 2011. Social Influences on Neurobiology and Behavior: Epigenetic Effects During Development. Psychoneuroendocrinology 36, 352–371. https://doi.org/10.1016/j.psyneuen.2010.06.005
9) Dief, A.E., Sivukhina, E.V., Jirikowski, G.F., 2018. Oxytocin and Stress Response. Open J. Endocr. Metab. Dis. 8, 93–104. https://doi.org/10.4236/ojemd.2018.83010
10) Dozio, E., Feldman, M., Bizouerne, C., Drain, E., Laroche Joubert, M., Mansouri, M., Moro, M.R., Ouss, L., 2020. The Transgenerational Transmission of Trauma: The Effects of Maternal PTSD in Mother-Infant Interactions. Front. Psychiatry 11. https://doi.org/10.3389/fpsyt.2020.480690
11) Dvorská, I., Brust, P., Hrbas, P., Rühle, H.J., Barth, T., Ermisch, A., 1992. On the blood-brain barrier to peptides: effects of immobilization stress on regional blood supply and accumulation of labelled peptides in the rat brain. Endocr. Regul. 26, 77–82.
12) Ermisch, A., Rühle, H.J., Landgraf, R., Hess, J., 1985. Blood-brain barrier and peptides. J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. 5, 350–357. https://doi.org/10.1038/jcbfm.1985.49
13) Gatta, E., Mairesse, J., Deruyter, L., Marrocco, J., Van Camp, G., Bouwalerh, H., Lo Guidice, J.-M., Morley-Fletcher, S., Nicoletti, F., Maccari, S., 2018. Reduced maternal behavior caused by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance. NeuroToxicology 66, 138–149. https://doi.org/10.1016/j.neuro.2018.04.005
14) Hanson, L.R., Frey, W.H., 2008. Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. BMC Neurosci. 9, S5. https://doi.org/10.1186/1471-2202-9-S3-S5
15) Hertzman, C., 1999. The biological embedding of early experience and its effects on health in adulthood. Ann. N. Y. Acad. Sci. 896, 85–95. https://doi.org/10.1111/j.1749-6632.1999.tb08107.x
16) Kenkel, W.M., Perkeybile, A.-M., Yee, J.R., Pournajafi-Nazarloo, H., Lillard, T.S., Ferguson, E.F., Wroblewski, K.L., Ferris, C.F., Carter, C.S., Connelly, J.J., 2019. Behavioral and epigenetic consequences of oxytocin treatment at birth. Sci. Adv. 5, eaav2244. https://doi.org/10.1126/sciadv.aav2244
17) Klengel, T., Dias, B.G., Ressler, K.J., 2016. Models of Intergenerational and Transgenerational Transmission of Risk for Psychopathology in Mice. Neuropsychopharmacology 41, 219–231. https://doi.org/10.1038/npp.2015.249
18) Lee, M.R., Shnitko, T.A., Blue, S.W., Kaucher, A.V., Winchell, A.J., Erikson, D.W., Grant, K.A., Leggio, L., 2020. Labeled oxytocin administered via the intranasal route reaches the

brain in rhesus macaques. Nat. Commun. 11, 2783. https://doi.org/10.1038/s41467-020-15942-1

19) Lev-Wiesel, R., 2007. Intergenerational Transmission of Trauma across Three Generations: A Preliminary Study. Qual. Soc. Work 6, 75–94. https://doi.org/10.1177/1473325007074167
20) Maccari, S., Piazza, P.V., Kabbaj, M., Barbazanges, A., Simon, H., Le Moal, M., 1995. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J. Neurosci. Off. J. Soc. Neurosci. 15, 110–116.
21) Maccari, S., Polese, D., Reynaert, M.-L., Amici, T., Morley-Fletcher, S., Fagioli, F., 2017. Early-life experiences and the development of adult diseases with a focus on mental illness: The Human Birth Theory. Neuroscience 342, 232–251. https://doi.org/10.1016/j.neuroscience.2016.05.042
22) Marrocco, J., Mairesse, J., Ngomba, R.T., Silletti, V., Van Camp, G., Bouwalerh, H., Summa, M., Pittaluga, A., Nicoletti, F., Maccari, S., Morley-Fletcher, S., 2012. Anxiety-Like Behavior of Prenatally Stressed Rats Is Associated with a Selective Reduction of Glutamate Release in the Ventral Hippocampus. J. Neurosci. 32, 17143–17154. https://doi.org/10.1523/JNEUROSCI.1040-12.2012
23) Meaney, M.J., 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu. Rev. Neurosci. 24, 1161–1192. https://doi.org/10.1146/annurev.neuro.24.1.1161
24) Moisiadis, V.G., Constantinof, A., Kostaki, A., Szyf, M., Matthews, S.G., 2017. Prenatal Glucocorticoid Exposure Modifies Endocrine Function and Behaviour for 3 Generations Following Maternal and Paternal Transmission | Scientific Reports. Sci. Rep. 7, 11814. https://doi.org/10.1038/s41598-017-11635-w
25) Morley-Fletcher, S., Gaetano, A., Gao, V., Gatta, E., Van Camp, G., Bouwalerh, H., Thomas, P., Nicoletti, F., Maccari, S., 2024. Postpartum Oxytocin Treatment via the Mother Reprograms Long-Term Behavioral Disorders Induced by Early Life Stress on the Plasma and Brain Metabolome in the Rat. Int. J. Mol. Sci. 25, 3014. https://doi.org/10.3390/ijms25053014
26) Neumann, I.D., Maloumby, R., Beiderbeck, D.I., Lukas, M., Landgraf, R., 2013. Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. Psychoneuroendocrinology 38, 1985–1993. https://doi.org/10.1016/j.psyneuen.2013.03.003
27) Parker, K.J., Buckmaster, C.L., Schatzberg, A.F., Lyons, D.M., 2005. Intranasal oxytocin administration attenuates the ACTH stress response in monkeys. Psychoneuroendocrinology 30, 924–929. https://doi.org/10.1016/j.psyneuen.2005.04.002
28) Riem, M.M.E., Bakermans-Kranenburg, M.J., Pieper, S., Tops, M., Boksem, M.A.S., Vermeiren, R.R.J.M., van Ijzendoorn, M.H., Rombouts, S.A.R.B., 2011. Oxytocin modulates amygdala, insula, and inferior frontal gyrus responses to infant crying: a randomized controlled trial. Biol. Psychiatry 70, 291–297. https://doi.org/10.1016/j.biopsych.2011.02.006

29) Riem, M.M.E., Bakermans-Kranenburg, M.J., van IJzendoorn, M.H., 2016. Intranasal administration of oxytocin modulates behavioral and amygdala responses to infant crying in females with insecure attachment representations. Attach. Hum. Dev. 18, 213–234. https://doi.org/10.1080/14616734.2016.1149872

30) Skinner, M.K., 2008. What is an epigenetic transgenerational phenotype? F3 or F2. Reprod. Toxicol. Elmsford N 25, 2–6. https://doi.org/10.1016/j.reprotox.2007.09.001

31) Takayanagi, Y., Onaka, T., 2022. Roles of Oxytocin in Stress Responses, Allostasis and Resilience. Int. J. Mol. Sci. 23, 150. https://doi.org/10.3390/ijms23010150

32) Tricker, P.J., 2015. Transgenerational inheritance or resetting of stress-induced epigenetic modifications: two sides of the same coin. Front. Plant Sci. 6, 699. https://doi.org/10.3389/fpls.2015.00699

33) van Steenwyk, G., Roszkowski, M., Manuella, F., Franklin, T.B., Mansuy, I.M., 2018. Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation. Environ. Epigenetics 4. https://doi.org/10.1093/eep/dvy023

34) Verhaeghe, R., Gao, V., Morley-Fletcher, S., Bouwalerh, H., Van Camp, G., Cisani, F., Nicoletti, F., Maccari, S., 2021. Maternal stress programs a demasculinization of glutamatergic transmission in stress-related brain regions of aged rats. GeroScience 44, 1047–1069. https://doi.org/10.1007/s11357-021-00375-5

35) Ward, I.D., Zucchi, F.C.R., Robbins, J.C., Falkenberg, E.A., Olson, D.M., Benzies, K., Metz, G.A., 2013. Transgenerational programming of maternal behaviour by prenatal stress 8.

36) Xu, G., Li, Y., Ma, C., Wang, C., Sun, Z., Shen, Y., Liu, L., Li, S., Zhang, X., Cong, B., 2019. Restraint Stress Induced Hyperpermeability and Damage of the Blood-Brain Barrier in the Amygdala of Adult Rats. Front. Mol. Neurosci. 12. https://doi.org/10.3389/fnmol.2019.00032

37) Yao, S., Chen, Y., Zhuang, Q., Zhang, Y., Lan, C., Zhu, S., Becker, B., Kendrick, K.M., 2023. Sniffing oxytocin: Nose to brain or nose to blood? Mol. Psychiatry 28, 3083–3091. https://doi.org/10.1038/s41380-023-02075-2

38) Yao, S., Kendrick, K.M., 2022. Effects of Intranasal Administration of Oxytocin and Vasopressin on Social Cognition and Potential Routes and Mechanisms of Action. Pharmaceutics 14, 323. https://doi.org/10.3390/pharmaceutics14020323

39) Yehuda, R., Bell, A., Bierer, L.M., Schmeidler, J., 2008. Maternal, not paternal, PTSD is related to increased risk for PTSD in offspring of Holocaust survivors. J. Psychiatr. Res. 42, 1104–1111. https://doi.org/10.1016/j.jpsychires.2008.01.002

40) Yehuda, R., Schmeidler, J., Labinsky, E., Bell, A., Morris, A., Zemelman, S., Grossman, R.A., 2009. Ten-Year Follow-Up Study of PTSD Diagnosis, Symptom Severity, and Psychosocial Indices in Aging Holocaust Survivors. Acta Psychiatr. Scand. 119, 25–34. https://doi.org/10.1111/j.1600-0447.2008.01248.x

**Supplementary Table 1.** Statistical analysis of correlation data among adult F3 offspring and their behavioral and endocrine correlates (Risk-taking behavior, OT, and CORT levels). Significance in bold.

| Interactions | r-value | p-value |
|---|---|---|
| F3 endocrinology | | |
| F3 CORT x F3 OT | 0.17 | 0.39 |
| F3 endocrinology x F3 behavior | | |
| F3 CORT x F3 risk-taking behavior | -0.26 | 0.16 |
| F3 OT x F3 risk-taking behavior | **0.37** | **0.05** |

**Supplementary Table 2.** Statistical analysis of correlation data among adult F3 offspring and their F2 mothers and F0 great-grandmothers correlates. Significance in bold.

| Interactions | r-value | p-value |
|---|---|---|
| F2 mothers x F3 offspring | | |
| F2 maternal care x F3 risk-taking behavior | **0.39** | **0.002** |
| F2 maternal reactivity x F3 risk-taking behavior | **-0.31** | **0.01** |
| F2 maternal CORT x F3 risk-taking behavior | 0.004 | 0.97 |
| F2 maternal OT x F3 risk-taking behavior | 0.17 | 0.23 |
| F2 maternal care x F3 CORT | -0.27 | 0.14 |
| F2 maternal reactivity x F3 CORT | 0.31 | 0.09 |
| F2 maternal CORT x F3 CORT | **0.44** | **0.01** |
| F2 maternal OT x F3 CORT | -0.25 | 0.17 |
| F2 maternal care x F3 OT | 0.30 | 0.12 |
| F2 maternal reactivity x F3 OT | -0.32 | 0.10 |
| F2 maternal CORT x F3 OT | 0.008 | 0.96 |
| F2 maternal OT x F3 OT | 0.24 | 0.12 |
| F0 great-grandmothers x F3 offspring | | |
| F0 maternal care x F3 risk-taking behavior | **0.48** | **0.0001** |
| F0 maternal reactivity x F3 risk-taking behavior | **-0.48** | **0.0001** |
| F0 maternal CORT x F3 risk-taking behavior | **-0.56** | **0.0001** |
| F0 maternal OT x F3 risk-taking behavior | 0.15 | 0.28 |
| F0 maternal care x F3 CORT | **-0.50** | **0.005** |
| F0 maternal reactivity x F3 CORT | **0.58** | **0.0007** |
| F0 maternal CORT x F3 CORT | 0.28 | 0.12 |
| F0 maternal OT x F3 CORT | -0.07 | 0.71 |
| F0 maternal care x F3 OT | **0.39** | **0.04** |
| F0 maternal reactivity x F3 OT | -0.32 | 0.10 |
| F0 maternal CORT x F3 OT | -0.31 | 0.11 |
| F0 maternal OT x F3 OT | ***0.35*** | ***0.06*** |

# Chapter Two: The anti-stress effect of *Limosilactobacillus reuteri* in mothers-offspring dyad

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## A. The corrective effects of *Limosilactobacillus reuteri* on maternal behavior via psychobiotic-induced oxytocinergic activity (*Article n° 3 submitted to Biological Psychiatry Global Open Science*)

Stressful events have been increasingly recognized for their significant impact on parental relationships, particularly maternal behavior (Gatta et al., 2018; Krol and Grossmann, 2018). There is mounting evidence suggesting that stress can disrupt the delicate balance of interactions within the mother-child dyad, and primarily impacting the mothers, leading to neurodevelopmental and psychiatric disorders, including *postpartum* depression (Maccari et al., 2017; Wang et al., 2018), thus influencing bonding and caregiving behaviors. Importantly, these effects are often mediated by oxytocin, an anti-stress hormone crucial for social bonding and maternal behaviors (Carter, 2014; Ivell et al., 2018).

In light of this understanding, this first section aimed to explore interventions that could potentially mitigate the negative effects of stress on parental relationships and maternal behavior. Recent research has shown that certain probiotics, such as *Limosilactobacillus reuteri*, have the ability to increase oxytocin levels (Buffington et al., 2016; Danhof et al., 2023; Dooling et al., 2022; Sgritta et al., 2019). Indeed, a study aimed at understanding the mechanisms behind *L. reuteri*'s effects revealed that its administration increased activation of oxytocin neurons, improved social behavior, and normalized social deficits in mouse models of autism, and those effects were not mediated by restoring the composition of the host's microbiome, but *via* the vagus nerve and induced synaptic plasticity in the ventral tegmental area of ASD mice, but not in oxytocin receptor-deficient mice (Sgritta et al., 2019) Additionally, the probiotic strain had an anti-stress effect by reducing CORT levels in the mice model, thus giving first evidence of anti-stress capacities of the probiotic (Varian et al., 2017). Similarly, Patterson and collaborators found that these specific lactobacilli strains positively influenced metabolism and reduced depressive-like behavior in the mice, suggesting potential therapeutic applications for probiotics in metabolic disorders and mood-related conditions (Patterson et al., 2019)

Therefore, within this context, we sought to investigate whether probiotic supplementation during the *postpartum* period in a stress model could restore maternal behavior and enhance oxytocin levels, both peripherally and in the hypothalamus, the brain structure implicated in oxytocin synthesis, indeed

very few studies focus on this structure to elucidate the mechanisms underlying maternal care and specifically probiotic implication in it.

Furthermore, our study aimed to elucidate the underlying mechanisms by which probiotics may exert these effects by evaluating peripheral hormones involved in the stress (Corticosterone) and anti-stress (Oxytocin) balance, as well as examining specific markers in the brain, such as Brain-Derived Neurotrophic Factor (BDNF). BDNF plays a pivotal role in maternal behavior (Maynard et al., 2018; O'Sullivan et al., 2011) and in neuronal development and synaptic plasticity, offering insights into how interventions like probiotic supplementation could potentially promote resilience during stressful events. By addressing these research questions, our study aims to contribute valuable insights into the therapeutic potential of probiotics in enhancing maternal behavior and preserving parental relationships amidst stressful conditions**.**

## Article n° 3 submitted to Biological Psychiatry Global Open Science

*Article n°3 submitted to Biological Psychiatry Global Open Science*

# *Limosilactobacillus reuteri* Supplementation in Lactating Rats Improves Maternal Behavior and Hypothalamic Oxytocin/ Brain-derived neurotrophic factor balance, Offering Insights for *Postpartum* Depression

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## ABSTRACT

Environmental challenges by disrupting parental care, have adverse effects on infant behavioral, emotional, and cognitive development, and predispose individuals to maladaptive responses and long-term health issues. Indeed, some new moms experience a long-lasting form of depression known as *postpartum* depression (PPD) which strongly impacts maternal care and maternal health. Here we addressed whether *postpartum Limosilactobacillus reuteri (L. reuteri)* treatment in lactating rats could reverse disrupted maternal care and neurobiological alterations induced by their gestational stress. Our results reveal that *L. reuteri* effectively mitigated pronounced behavioral changes triggered by maternal stress, including decreased maternal care, and diminished reactivity to pups' separation, without affecting non-pups-oriented behaviors. Crucially, *L. reuteri* demonstrated modulatory effects on the oxytocin/Brain-Derived Neurotrophic Factor (BDNF) balance in the hypothalamus, evidenced by the reduction of elevated BDNF levels and an increase in oxytocin (OT) levels in stressed dams adjusting oxytocin receptor (OTR) forms in treated dams under stress. This study showed for the first time the therapeutic role of *L. reuteri* in alleviating the adverse effects of gestational stress on maternal behavior and physiology, providing insights into its molecular mechanisms and offering potential avenues for bio-pharmacological interventions that could highly improve maternal care in the context of PPD.

**Keywords**: Gestational stress, *postpartum* maternal care, *postpartum* depression, *L. reuteri*, oxytocin and oxytocin receptor, BDNF, maternal health.

## INTRODUCTION

Stressors during pregnancy can significantly impact maternal care, influencing child development and programming maladaptive responses that may lead to long-term health issues (Duffy et al., 2024; Maccari et al., 2017). Moreover, in the critical *peripartum* period, some new moms experience a long-lasting form of depression known as *postpartum* depression (PPD). PPD is a serious psychiatric disorder (Payne and Maguire, 2019) that is understudied (both clinically and experimentally) and underdiagnosed with strong impacts on both the maternal care of the offspring and maternal health (Feldman et al., 2009; Righetti-Veltema et al., 2003).

Early life stress (ELS) in preclinical animal models (Maccari et al., 2014; Weaver et al., 2004) and in humans (Turecki and Meaney, 2016), results from intricate interactions between genes and the environment, involving epigenetic mechanisms transmitted through alterations in maternal care. Indeed, naturally occurring variations in maternal care have been associated with increased methylation of the glucocorticoid receptor promoter, linked to prolonged corticosterone (CORT) secretion in response to stress in the adult offspring of mothers providing low levels of licking (Meaney, 2010). Notably, enhancing maternal care, such as through early adoption, has been demonstrated to reverse the effects of perinatal stress (PRS) on the impaired corticosterone negative feedback in adult offspring (Maccari et al., 1995). In the PRS rat model, increased maternal glucocorticoids and decrease oxytocin signaling induced by gestational stress reduce maternal care (Gatta et al., 2018) and disrupt the hypothalamic-pituitary-adrenal (HPA) axis activity in offspring (Barbazanges et al., 1996; Maccari et al., 1995). *Postpartum* treatment with carbetocin, an oxytocin (OT) analog activating the OT receptor (OTR), restores maternal care reduction in stressed dams and corrects maladaptive programming in the PRS adult offspring rat (Gatta et al., 2018; Morley-Fletcher et al., 2024).

The hypothalamic neuropeptide OT is pivotal in shaping maternal behavior, evident in both animal models and human studies linking perturbed OT transmission to poor maternal behavior (Sanson and Bosch, 2022). In humans, inadequate mothering is associated with defective OT signaling both in the brain and blood (Toepfer et al., 2019), associated with reduced OT gene expression and paralleled by increased methylation of the OT promoter during pregnancy (Toepfer et al., 2019). Individual differences in maternal behavior in rats are also linked to changes in OTR levels in brain regions mediating maternal care (Champagne et al., 2001; Champagne and Meaney, 2006), and in humans, OTR regulates complex social behaviors such as parenting and responses to stress (Cataldo et al., 2018). In addition to its known role in maternal behavior and social bonding, there is growing evidence suggesting the involvement of the OT/OTR system in modulating brain plasticity and psychiatric disorders (Triana-Del Rio et al., 2022). Recent studies have highlighted an intricate interplay between OT and BDNF, a crucial marker of neuroplasticity reduced by high levels of CORT.

Animal research indicates that OT may boost the expression of BDNF genes, along with their levels in the rat hippocampus (Bukatova et al., 2023; Havranek et al., 2015) associated with a reduction of CORT. Regarding maternal behavior, genetic investigations in rats focusing on BDNF have revealed its significant expression in the hypothalamus and underscored the role of the BDNF/TrkB signaling in OT hypothalamic neurons, emphasizing its contribution to maternal behavior (Maynard et al., 2018). Indeed, the inactivation of the BDNF gene has been found to impair maternal care in female rats, concurrently reducing OT transcripts in OT neurons.

Gestational stress and abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis have been linked to PPD. Indeed, HPA axis abnormalities are considered strong biomarkers that can be combined with clinical observation for a better PPD diagnosis (Rathi et al., 2022). In accordance with the clinical evidence for stress as a risk factor for PDD, many of the animal models used to study PPD utilize exogenous corticosterone- or stress-based models (Zhu et al., 2023). However, the molecular changes in the brain that contribute to this pathological condition in a crucial period of life for both newborns and new moms remain largely unknown, which create a pressing need for specific molecular biomarkers such as OT, oxytocin receptors (OTR) and BDNF (Asherin et al., 2020; Singh et al., 2023; Zhu et al., 2023) which have been involved in preclinical animal model of ELS characterized by reduced maternal care (Maynard et al., 2018; Roth et al., 2009).

The OT/OTR system exhibits anti-inflammatory properties and plays a crucial role in gut-brain communication (Buffington et al., 2016; Sgritta et al., 2019). It exerts protective effects on the gastrointestinal system in experimental models of colitis and in human subjects with gastric injury and colitis (Kingsbury and Bilbo, 2019). Conversely, gut-brain axis alterations are compromised by chronic stress (Lee et al., 2021; O'Mahony et al., 2020). Furthermore, OT directly impacts the composition of the gut microbiome (Barengolts et al., 2018) and microbiome composition stimulates OT release from the intestinal epithelium via secretin signaling across various species, including humans (Danhof et al., 2023). In this regard, probiotics have demonstrated therapeutic potential in modulating the gut-brain axis, ameliorating gut dysfunction induced by stress, and influencing social behavior (Codagnone et al., 2019; Gareau et al., 2007; Sgritta et al., 2019).

Of relevance, *Limosilactobacillus reuteri* (*L. reuteri*) ATCC PTA 6475, originally isolated from human breast milk, exhibits OT-activating properties in various contexts (Buffington et al., 2016; Poutahidis et al., 2013; Varian et al., 2017). However, it is unknown whether *L. reuteri* can increase maternal care despite its role in regulating OT levels. Recent research on another *L. reuteri* strain, PBS072, has proven effective in improving mothers' moods, in association with enhanced breastfeeding quality, and reduced babies' crying, though it did not analyze mother-infant interaction (Vicariotto et al., 2023).

Moreover, the neurobiological influence of hypothalamic BDNF levels via OT to improve maternal care is unknown and it is of crucial importance to reinforce the treatment of *L. reuteri* in clinic use.

Here we used *postpartum* treatment with *L. reuteri* ATCC PTA 6475 in dam rats which were exposed to gestational stress in order to restore maternal care and explore potential molecular mechanisms of *L. reuteri*. Our findings indicate, for the first time, that *L. reuteri* supplementation increases maternal OT levels in both plasma and the brain, thereby enhancing maternal care through its influence on the OT-BDNF interplay in the hypothalamus. This preclinical study provides insights into the molecular mechanisms of *L. reuteri* offering potential avenues for bio-pharmacological interventions that could positively influence maternal health trajectories in the context of PPD, of the new moms in a critical period of development of the newborns.

## MATERIALS AND METHODS

### Ethics

All experiments followed the rules of Directive 2019/10/10 of the Council of the European Communities and the Comité d'Ethique CEEA-75 (Comité d'Ethique en Expérimentation Animale Nord-Pas de Calais). This project was approved by the MESRI (Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation ; authorization #33654).

### Animals

25 nulliparous Sprague Dawley female rats (Charles River, France) weighing approximately 250 g, were purchased from Charles River (France) and housed under standard conditions with a 12 h light/dark cycle (lights on 7 am: lights off 7 pm). Upon arrival in the animal facility, the animals were placed in large cages (between 3 to 5 animals per cage) for 10 days to acclimatize and synchronize their estrous cycle. After group housing (five females/cage) for two weeks, each female was individually housed for one week with a sexually experienced male rat. Following that, a gain of at least 10 grams was considered as an index of pregnant status. Pregnant females were then randomly assigned to either the stress or the control group.

### Gestational stress procedure

14 pregnant rats underwent the gestational restraint stress procedure following our standard protocol (Maccari et al., 1995), while the remaining females were left undisturbed (control unstressed group). Starting from the 11th day of pregnancy until delivery, the dams experienced three daily stress sessions, each lasting 45 minutes. There was a minimum 2-hour interval between each stress session. Control pregnant females were left undisturbed in their home cages and were handled once per week. During gestation, the weight of the rats was monitored weekly. Weaning took place 21 days after

birth. Dams's body weight monitoring also occurred during the *postpartum* period (PP) at PP7 and PP21. The collected data were normalized to control unstressed dams -water treated with the values represented as percentages (%).

**Culture and treatment with *L. reuteri* during lactation *postpartum***

To modulate the OT system, lactating dams were given a probiotic treatment with *Limosilactobacillus reuteri ATCC PTA 6475* (BioGaia AB, Eslöv, Sweden) during the first *postpartum* week (PP1-PP8). *L. reuteri* was cultured anaerobically in MRS broth (Millipore) and assessed on MRS agar. Cultures were centrifuged, washed, resuspended in Phosphate-Buffered Saline (PBS), and frozen at -80°C until use the following week. The live *L. reuteri* was added to the drinking water at a dosage of 1 $x10^8$ CFU/ml (colony forming unit/ml). The first group of dams received live *L. reuteri* (*L. reuteri* group), while the second group received PBS-treated water (water group). Both groups consumed the treated water *ad libitum*, for the probiotic group the total dose consumed was between 0.78 x $10^{10}$ to 0.79 x $10^{10}$ CFU/rat per day.

**Experimental design**

The experimental design and timeline are summarized in **Figure 1A**. The probiotic treatment was administered during the 1st *postpartum* week from PP1 to PP8. This experiment included 4 different groups combination; Stressed mothers treated with probiotics (STRESS+ *L. reuteri*; n=8 rats/group) or water (STRESS+ water; n=6 rats/group), and the corresponding control unstressed groups (CONT+ *L. reuteri* or CONT+ water; n=5 rats/group and n=6 rats/group, respectively). Dams were tested for maternal care as well as for their response to pups' separation during the first lactation week. Immediately after weaning of the pups, the dams were tested for their exploratory and risk-taking behavior in the EPM (Elevated-Plus Maze) as well as for locomotor reactivity to novel environments in the actimiter. Subsequently, brain and blood tissues were collected for endocrine and biochemical analysis. At the end of the experimentation, mothers were 3 to 3.5 months old.

**Pup-directed behaviors during lactation**

Maternal care. was constantly monitored every day during the first week of lactation. with small infrared cameras (AMC, France) placed on the animal cage rack where cages containing lactating females were placed. The active behavior of the mother in the nest (nursing behavior, licking, carrying pups, and arched back over pups) was scored every minute (60 observations/h with 1 h of observation per day, from 7 am to 8 am) and the data obtained were expressed as a percentage of maternal care with respect to the total number of observations, thus allowing for a comprehensive understanding of maternal response to the gestational stress and *L. reuteri* treatment (Morley-Fletcher et al., 2024). Maternal response to pups' separation test. In addition to the observation of natural home-cage

behavior, maternal reactivity following a 15-minutes separation from pups on PP7 was also investigated. During the separation, the weights of the mothers and pups were recorded, and the sex of the pups was identified. After reuniting, the latency to initiate contact with the pups was measured, and the time spent nursing or in self-directed behavior were recorded for 10 minutes.

**Non-pup-directed behaviors after weaning**

***Locomotor reactivity to novel environment***. At PP 22, animals were assessed for their locomotor reactivity to a novel environment in the actimeter (Imetronic, Bordeaux, France). The actimeter was equipped with infrared sensors (beams) for locomotor activity detection, a removable cage with a feeding trough, and a water bottle. Each female was placed in one of the actimeter cages for 90 minutes. The data were monitored for 90 minutes. The amount of locomotor activity is the sum of rearrings and back-and-forth movements and were represented by the frequency of infrared sensor interruptions. ***Exploratory activity and risk-taking behavior in the elevated-plus maze (EPM)***. At PP 24, the behavior of rat dams was assessed in the elevated-plus maze (EPM) according to our previous protocol (Marrocco et al., 2012) with a video camera tracking using specific software (EthoVision, Noldus, EthoVision, The Netherlands). The number of visits and time spent in each arm were measured. The exploratory behavior was measured through the number of visits in both the open and closed arms, and risk-taking behavior as the presence of the rat in the open arms (visits, time spent and latency).

**Endocrine analysis**

Plasma assessment of OT and CORT levels was performed in trunk blood samples collected around PP30 with rat/ELISA kit (OT, sensitivity 9.4 pg/ml-CUSABIO # CSB-E14197r; CORT, sensitivity 6.1 ng/mL-Demeditec # DEV9922). OT levels in the hypothalamus were measured by ELISA test (Enzo ADI-901-153A; sensitivity 15 pg/ml.) which was performed in the supernatant obtained after centrifugation (15,000 x g, 20 min, 4°C) of homogenized hypothalamic tissue in HEPES-buffered sucrose. Results were normalized to protein quantity after a BCA assay.

**Biochemical analysis**

Measurement of OTR and BDNF protein expression in the hypothalamus by western blotting was performed in crude synaptosomal fractions according to a previous established protocol (Morley-Fletcher et al., 2018) using antibodies against BDNF (1:500, #282051-AP, Proteintech), and oxytocin receptor (OTR) (1:250, #sc-33209, Santa Cruz). Anti-BDNF detected the precursor BDNF (proBDNF), the truncated BDNF (tBDNF) and the mature (mBDNF) isoforms (32, 28 and 14kDa respectively), while anti-OTR detected both the native (45kDa) and glycosylated (55kDa) isoforms.

Data were normalized to the expression of β-actin (1:1000, #A5316, Sigma) and then expressed as a ratio to the CONT water dams' group.

**Statistical analyses**

Statistical analyses were conducted using STATISTICA 8.0 (Stat Soft. Inc). The normality of data distribution was assessed using the Shapiro-Wilk test, and then a Two-ways ANOVA with Newman-Keuls post-hoc (NK) or Planned Comparisons (p.c) tests were applied. Independent variables included group* with Stress (stress *vs.* cont) and probiotic# (*L. reuteri vs.* water). Pearson correlations analysis were used to investigate association between behavioral, endocrine and neurochemical parameters. A p-value < 0.05 was considered significant. All graphs were created with GraphPad PRISM Version 10.2.3.

**RESULTS**

***L. reuteri* reduced weight gain during the *postpartum* period independently of the stress**

At delivery of the offspring, we monitored the body weight of the rat dams on days PP7 and PP21. Stress had no effect while *L. reuteri* (**Figure 1B**) significantly reduced body weight in both PP7 and PP21 in Stress and unstressed groups (n=5-8 rats/group rats/group; *probiotic effect*, $F_{(1, 21)}$=4.55, p=0.04#). At G14 and G20, stress to mothers previously impacted body weight **(supplementary S1A**).

**Appetitive value of *L. reuteri postpartum* administration**

We observed an increase of probiotic consumption with respect to water intake during the first *postpartum* week, independently from exposure to gestational stress (n=5-8 rats/group, probiotic consumption effect $F_{(1,21)}$=8.27, p=0.009##; average/week, Figure 1C). However, the daily analysis during the 7-days treatment period revealed that, within the stressed group, *L. reuteri* intake was higher compared with the corresponding water-treated group (*Stress x treatment x day interaction*, F $_{(6,126)}$=2.13, p=0.05 *#$; p.c. ##p<0.01 or #p<0.05).

***L. reuteri* corrected pups-directed behaviors during lactation in stressed mothers**

*Maternal care:* Gestational restraint stress reduced maternal care (licking/nursing amount) and *L. reuteri* rescued that deficit (n=5-8 rats/group, *Stress x probiotic interaction* $F_{(1,21)}$=10.49, p=0.003**/##; NK Stress *vs.* Cont in water groups, p=0.023*; Stress + water *vs.* Stress +*L. reuteri*, p=0.021#; **Figure 1D**). *Maternal reactivity:* When analyzing the maternal response to a 15-minute pups's separation (**Figure 1E-G**) on PP7, a PRS x probiotic interaction revealed that water stress dams had a longer latency (s) to do the first contact with the pups compared to water control dams,

and this was corrected by *L. reuteri* (n=5-8 rats/group, *Stress x probiotic interaction* $F_{(1, 21)}$=7.63, p=0.01**/##; NK, Stress *vs.* Cont in water group p=0.002**; water *vs. L. reuteri* in Stress group p=0.004##; **Figure 1E**). During the 10 minutes following the pup's reunion, we measured mother's nursing (**Figure 1F)** and self-directed behaviors (grooming and eating, **Figure 1G**). As gestational stress had no impact on these parameters, we observed a main effect of *L. reuteri* which increased nursing behavior and reduced self-directed behavior in both stressed and control groups (n=5-8 rats /group; *probiotic effect*, nursing, $F_{(1,21)}$=4.58, p=0.044#; self-directed behavior, $F_{(1,21)}$=4.98, p=0.03#). Altogether, maternal care and licking latency were strongly associated as identified by the negative correlation (r=-0.51, p=0.01**, **Figure 1H**), with *L. reuteri* increasing maternal care and reducing licking latency in stressed dams.

**Effect of *L. reuteri* on non-pups-directed behaviors**

Following the weaning of the pups, we assessed rat dams in non-pups-directed behaviors such as reactivity to novel environment and risk-taking and exploratory behavior. *Locomotor reactivity*: we analyzed on PP22 the locomotor reactivity of the dams to a novel environment in the actimeter **(Figure 1I)**. Neither stress nor the probiotic had an impact on total locomotor reactivity (n=5-8 rats/group; *stress × probiotic interaction*, $F_{(1,19)}$=0.75, p=0.39). *Exploratory activity and risk-taking behavior in the EPM*: At PP24 (**Figure 1J**), neither stress nor the probiotic had an impact in this behavioral test. Indeed, levels of activity (visits in the open/closed arms), as well as risk-taking behavior (time spent in the open arms, latency to enter the open arms and visits to open arms), were not different among the groups (n= 5-8 rats/group; total visits, *stress × probiotic interaction*, $F_{(1,20)}$=0.32, p=0.57; visits in the open arms, *stress × probiotic interaction*, $F_{(1,20)}$=0.41, p=0.71; risk-taking behavior*, stress × probiotic interaction*, $F_{(1,20)}$=0.13, p=0.71; latency to the open arms, *stress × probiotic interaction*, $F_{(1,20)}$=0.24, p=0.62).

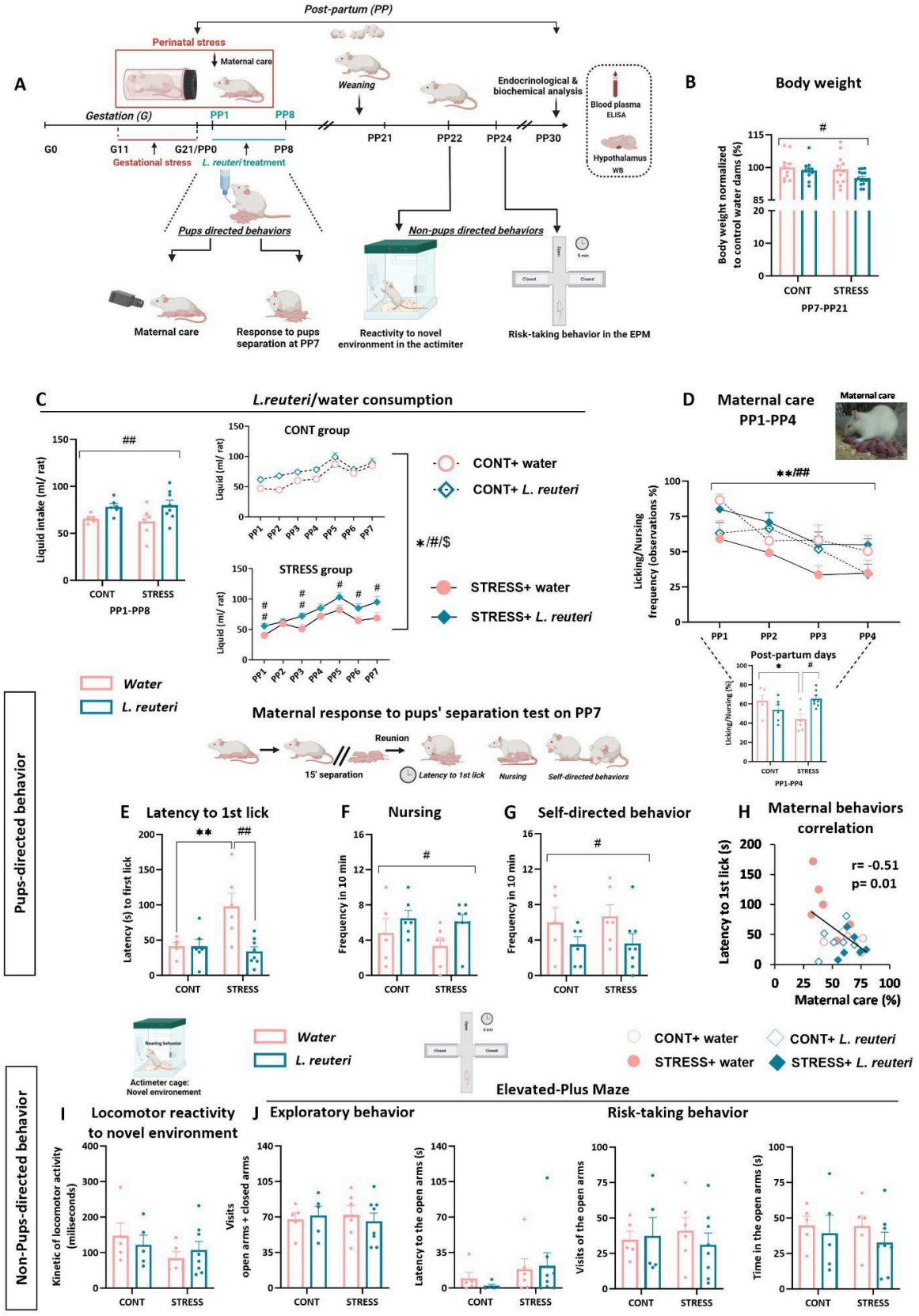


**Figure 1. Gestational stress and *postpartum L. reuteri* impacted pups-directed behaviors but had no effects on non-pups-directed behavior**

Experimental design (**A**). *L. reuteri* decreased weight gain (g) during the *postpartum* period independently of the stress groups, (n=5-8 rats/group) (**B**). Stressed female rat dams exhibited more liquid consumption with *L.*

*reuteri* over the 7 days treatment (ml/ rat) (n=5-8 rats/group) (**C**), . Gestational stress reduced maternal care *via* the reduction of licking/nursing during PP1-PP4, while *L. reuteri* given to stressed dams corrected it (**D**). For maternal response to pups' separation on PP7, gestational stress increased latency to lick which was reduced by *L. reuteri* (**E**), the probiotic increased nursing behavior (**F**) and reduced self-directed behaviors in both groups (**G**). Maternal care and latency to first lick in the maternal response to pups' separation test were correlated (**H**) At PP22, both gestational stress and *postpartum L. reuteri* did not impact the reactivity to the novel environment in the actimeter (**I**). At PP24, gestational stress and *postpartum L. reuteri* did not influence neither exploratory nor risk-taking behavior in the EPM (**J**). All values are means ± S.E.M. (n=5-8 rats/group), * $p < 0.05$ and **$p < 0.01$ for CONT vs. STRESS; and # $p < 0.05$ and ## $p < 0.01$ for water *vs*. *L. reuteri* corresponding group. For interaction effects; **/## < 0.01 for Stress *vs*. probiotic interactions and */#/$ $p < 0.05$ for Stress, probiotic, and day interactions.

**Impact of maternal stress and correction of *postpartum L. reuteri* on the stress (CORT) /anti-stress (OT) balance and the oxytocinergic /BDNF balance in plasma and the hypothalamus**

The experimental design is presented in **Figure 2A**. Stressed-water dams presented higher levels of plasma CORT and lower levels of OT compared to control unstressed dams which were restored to control levels by *L. reuteri* (**Figure 2B** n=5-6 rats/group, *stress x probiotic interaction;* CORT, $F_{(1,19)}$=15.88, p=0.0007***/###; NK, Stress *vs.* Cont in water groups, p=0.0001***; water *vs. L. reuteri* in the Stress group, p=0.0002###; OT levels, *stress* × *probiotic interaction*, $F_{(1,19)}$=12.90, p=0.002***/###; NK, Stress *vs.* Cont in water groups, p=0.05*; water *vs. L. reuteri* in the Stress group, p=0.0004###). Consequently, when analyzing the stress/anti-stress balance, we identified a significant negative correlation between plasma OT and CORT (r=-0.51, p=0.01**, Figure **2B**), which was ameliorated by the *L. reuteri* administration. Stressed-water dams presented lower levels of OT in the hypothalamus which were restored to control levels by the probiotic. *L. reuteri* increased OT levels in stressed mothers while it reduced it in controls (**Figure 2B;** n=5-6 rats/group, *stress* × *probiotic interaction*, $F_{(1,19)}$=50.48, p=0.000001***/###; NK, Stress *vs.* Cont in water groups, p=0.0008***; water *vs. L. reuteri* in the Stress group, p=0.0006###, and p=0.0002### in the Cont group). However, no significant association was found between hypothalamic OT levels and CORT. When looking the OTR protein expression in the hypothalamus, we found that gestational stress had an opposite effect in both isoform of OTR, since it reduced the 45kDa OTR isoform while it increased the 55kDa-glycosylated one (**Figure 2D**). Interestingly, OTR levels in stressed dams were further reduced by *L. reuteri* in both native and glycosylated isoforms, while in the control group *L. reuteri* reduced the native form and it increased the glycosylated form (45kDa OTR, n=6 rats/group, *stress* × *probiotic interaction*, $F_{(1,20)}$=25.44, p=0.00006***/###; NK, Stress *vs.* Cont in water groups, p=0.0002***; water *vs. L. reuteri*, p=0.04# in the Stress group and p=0.0001### in the Cont group; glycosylated 55kDa OTR, n=6 rats;/group *Stress* × *probiotic interaction* $F_{(1,20)}$=8.73, p=0.007**/##; NK, water *vs. L. reuteri* in Cont group p=0.008; Stress *vs.* Cont in *L. reuteri* groups, p=0.008). Finally, the total OTR assessed by combining the 45KDa and 55KDa forms revealed reduced levels in the stress group, as well as a decline in receptor levels in the *L. reuteri*-treated group (**supplementary**

**S1B**). Both native and glycosylated OTR forms were correlated with hypothalamic OT, with a positive correlation for OTR 45KDa and a negative correlation for glycosylated OTR 55kDa (**supplementary S1C**). No associations were observed between OTR and the stress anti-stress balance in the plasma as previously observed for hypothalamic OT (**extended data in Table 1-supplementary**). When measuring BDNF (**Figure 2E**), we observed higher levels of both proBDNF and mBDNF isoforms in stressed mothers, which were reduced by *L. reuteri* (n=6 rats/group; proBDNF, *stress* × *probiotic interaction*, $F_{(1,20)}$=9.72, p=0.005**/##; Stress *vs.* Cont in water groups, p=0.02; water *vs. L. reuteri* in the Stress group, p=0.004##); mBDNF, *stress* × *probiotic interaction*, $F_{(1, 20)}$=10.04, p=0.004**/##; NK, Stress *vs.* CONT in water groups, p=0.01; water *vs. L. reuteri* in the Stress group, p=0.01). A similar profile was observed for the tBDNF (**supplementary S1D**). In general *L. reuteri* had no effect on proBDNF nor on mBDNF expression of control unstressed dams. Consequently, the ratio proBDNF/mBDNF was reduced by *L. reuteri* in the stressed group (*probiotic effect*, $F_{(1,20)}$=3.39, p=0.08; p.c. water *vs. L. reuteri* in the Stress group, p=0.03#). This time, BDNF isoforms exhibited specific associations with the CORT/OT balance (**Figure 2F**). Indeed, ProBDNF was partially associated with the stress / antistress balance since it showed a negative correlation with plasma OT only (r=-0.51, p=0.001) while mBDNF displayed a complete association with the stress/anti-stress balance since it positively correlated with CORT (r=0.57, p=0.003**), and negatively with both plasma and hypothalamic OT (plasma OT: r=-0.64, p=0.0009***; hypothalamic OT: r=-0.53, p=0.007***)(**Figure 2F**).

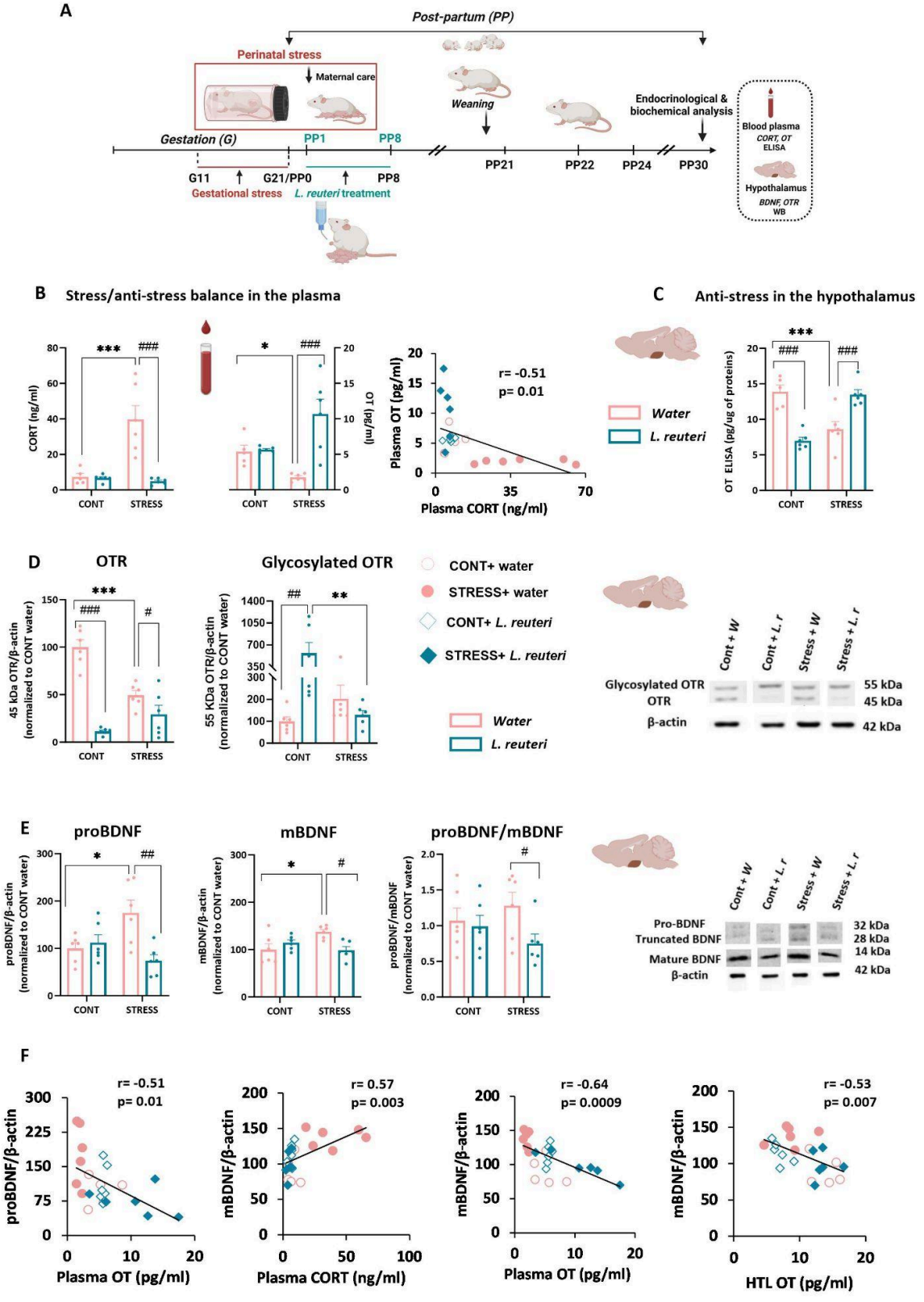


**Figure 2. Impact of maternal stress and correction of *postpartum L. reuteri* on the stress (CORT) /anti-stress (OT) balance and the oxytocinergic /BDNF balance in plasma and the hypothalamus** Experimental design (**A**). Gestational stress disrupted the plasmatic stress (CORT)/anti-stress (OT) balance and *postpartum L. reuteri* restored it (**B**). Gestational stress reduced OT levels in the hypothalamus (**C**), subsequently leading to an impact on native and glycosylated OTR (**D**) and on proBDNF, mBDNF and

proBDNF/mBDNF protein expression in the synaptosomal fraction of hypothalamus (**E**). *L. reuteri* increased OT levels and reduced OTR and BDNF isoforms. Pearson's correlations showed that proBDNF and mBDNF correlated with the stress/anti-stress balance (**F**). All values are means ± S.E.M. (n=6 rats/group), * $p < 0.05$,** $p < 0.01$ and ,*** $p < 0.001$ for CONT *vs.* STRESS, and # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ for water *vs. L. reuteri* corresponding group.

**Maternal behaviors correlate with the BDNF in the hypothalamus and with the CORT/OT balance in plasma**

We then investigated the associations between maternal behaviors (maternal care: nursing/licking; maternal responsivity: latency to lick pups following reunion in the separation test) and the OT/BDNF as well as the stress/anti-stress (CORT/OT) balance (measured in plasma and hypothalamus for OT), (**Figure 3**; **extended data in Table 1-2 supplementary**). No correlations were observed between maternal behaviors and OT levels in the brain, nor with OTR (both native and glycosylated forms). Conversely, BDNF exhibited specific associations with maternal behavior, with ProBDNF being correlated with both maternal behaviors (**Figure 3A-B**: maternal care, r=-0.47, p=0.02; maternal responsivity, r=0.65, p=0.007) while mBDNF was specifically associated with maternal responsivity to pups' separation (**Figure 3C** r=0.62, p=0.001).

Of note, plasma OT was specifically correlated with maternal care behavior (**Figure 3D,** r=0.46, p=0.02), but this specificity was not observed in the licking latency. This parameter, on the other hand, exhibited strong correlations with opposite profiles for both components of the stress/anti-stress balance, (**Figure 3E-F**; CORT r=0.48, p=0.01; OT, r=-0.54, p=0.007). In all significant associations identified, the Stress + water and Stress + *L. reuteri* groups were distinctly clustered in opposite directions on the scatterplot, indicating the restorative effect of *L. reuteri* in the stressed group.

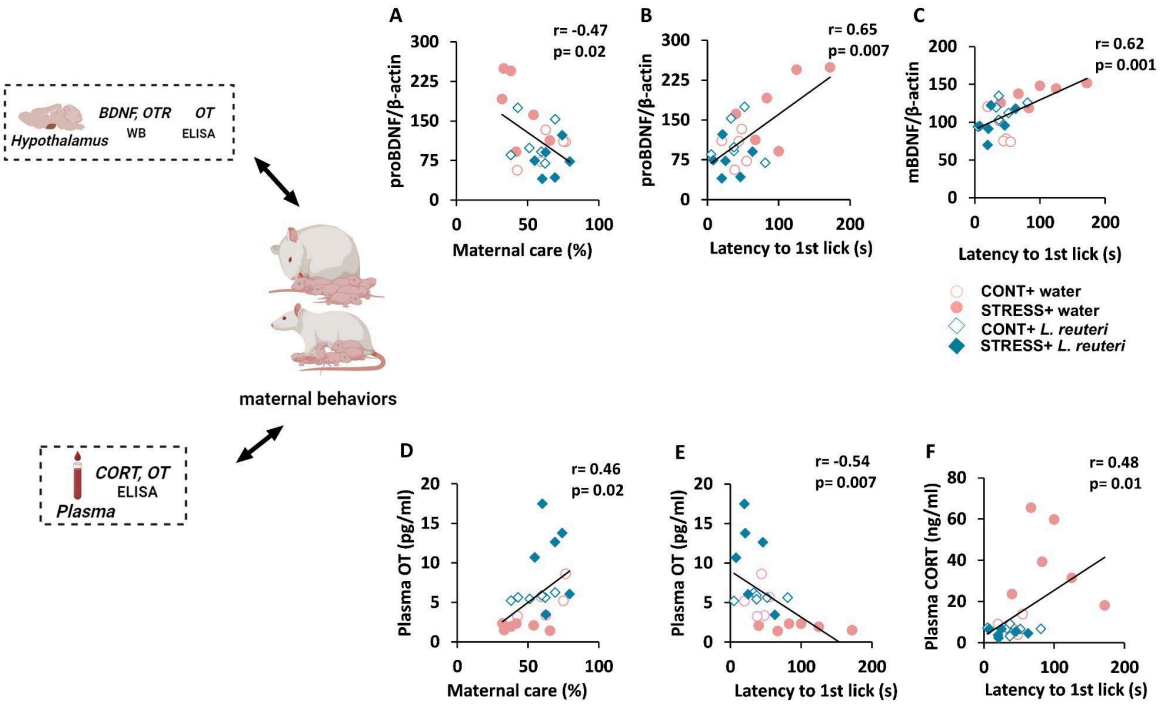


**Figure 3. Correlation analysis of maternal care (nursing/licking behavior during the first *postpartum* week) and licking latency in the separation test with BDNF in the hypothalamus and the stress /anti-stress balance in the plasma.** Pearson's correlation coefficient (r) values and related p values are reported in Supplementary Table 1 (n=5-6 rats /group).

## DISCUSSION

Our study explored in lactating rats the potential of short (1-week) *postpartum* treatment with *Limosilactobacillus reuteri* (*L. reuteri*) ATCC PTA 6475 to reverse maternal behavioral impairment illustrating its mechanisms of action at both brain and plasma levels. Here we present groundbreaking findings demonstrating, for the first time in lactating rat mothers, that *L. reuteri* positively influences maternal behavior, while both gestational stress and *L. reuteri* treatment had no effects on non-pups-oriented behaviors. This occurs by regulating the delicate balance between stress and anti-stress responses, through a specific enhancement of the OT/OTR system and by restoring the disrupted OT/CORT and BDNF balance caused by gestational stress in a key brain region of maternal care regulation as the hypothalamus. This investigation advances our understanding of molecular markers of the maternal environment. Moreover, it highlights a novel potential probiotic intervention for *postpartum* depression (PPD). Indeed probiotics, also known as psychobiotics (Dinan et al., 2013) have never been considered for their psychotropic effects in this specific psychiatric pathology that impacts a critical period of life both for the new mom and the newborn.

Notably, *L. reuteri* significantly reduced dams body weight during lactation, regardless of stress exposure. These findings support *L. reuteri*'s OT-like action, particularly its anorexigenic effect in

lactating dams, as previously demonstrated in stressed mothers treated with carbetocin (Gatta et al., 2018). Furthermore, these findings align with mounting evidence indicating weight loss and metabolic disorder mitigation following probiotic supplementation, notably with *L. reuteri* ATCC PTA 6475 and related strains (Crovesy et al., 2017; Liu et al., 2022; Lopes et al., 2023). On the other hand, as clinical investigations indicate weight gain in PPD patients (Herring et al., 2008), this underscores probiotics' potential for preventing or managing stress-related metabolic disorders and associated complications. Additionally, we observed increased liquid consumption in the *L. reuteri* group with respect to the water group, thus suggesting an appetitive value of *L*. reuteri. This appetitive quality reinforces the pleasant and non-invasive properties of *L. reuteri* in lactating dams. This was particularly evident in the stressed group, which benefited from the *postpartum* probiotic treatment. Then, the high appetitive value of *L. reuteri* could lead to positive outcomes by facilitating its administration due to its palatability, thereby enhancing the probiotic's effectiveness.

In stressed dams, we observed impaired pups-directed behaviors; including reduced maternal care and reduced maternal reactivity during brief pups' separation, which were correlated with elevated levels of CORT and decreased levels of OT. On the other hand, there was no significant impact on non-pups-directed behaviors, which makes a relevant breakthrough that mimics PPD-related deficits. Specifically, we observed that maternal stress-induced reductions in plasma OT levels were paralleled by similar alterations in OT levels and OTR protein expression within the hypothalamus. All these alterations were counteracted by *L. reuteri*. While previous research has established that increased CORT levels diminish maternal care (Angelucci et al., 1985; Zoubovsky et al., 2020), our results provide additional evidence for the positive regulatory role of the OT system in promoting maternal behavior (Gatta et al., 2018; Sanson and Bosch, 2022). OT was specifically correlated with maternal care behavior, while this specificity was not reflected in the licking latency parameter, which exhibited correlations with both components of the stress/anti-stress balance. Of note, there is evidence showing strong negative correlations between OT levels and PPD symptoms indicating that PPD moms present a reduction in plasma OT (Rathi et al., 2022; Thul et al., 2020; Zhu et al., 2023).

Interestingly, we observed a strong association between the forms of OT and its receptor (OTR) in the hypothalamus. Specifically, the native form of OT (45 kDa) showed a positive correlation, while the glycosylated form was negatively correlated with OT levels. In line with the observed low OT levels and high CORT levels, gestational stress resulted in a reduction of the 45 kDa non-glycosylated form of maternal OTR, while it increased the glycosylated form (55 kDa) of OTR in the hypothalamus. This extends previous observations showing diminished OTR protein levels in the medial preoptic area (MPOA) of low-licking rat mothers(Champagne et al., 2001) using autoradiography, although radiolabeling does not allow for discrimination of specific OTR isoforms. Thus, our finding marks the first evidence of OTR protein expression and its isoforms in the stressed maternal brain during

gestation. The supplementation of *L. reuteri* by enhancing OT levels in the brain exhibited nuanced effects on OTR expression. It reduced OTR expression in both groups in its native form while increasing the glycosylated form in controls and decreasing it in stressed dams. This variable modulation of different OTR forms by gestational stress, coupled with the significant reduction of OTR by *L. reuteri,* suggests a potential desensitization effect induced by the increased release of OT is facilitated by the probiotic, which would in turn induce less responsivity to further OT stimulation in the hypothalamic neuronal cells (Robinson et al., 2003). Interestingly, increased DNA methylation of the OTR gene promoter in the blood has been linked to risk of PPD (Bell et al., 2015), in association with OTR gene expression deficits and rs53576 OTR polymorphisms. Similarly in our study, reduced OTR protein level between control and stressed dams was highlighted, even though *L. reuteri* did not increase OTR protein abundance in the HTL as expected, but that could be explained as a desensitization mechanism. Overall, this underscores the efficacy of a short treatment with *L. reuteri* in activating the OT system in the lactating dam. This specific effect of *L. reuteri* on the OTR lies in its potential to deepen our understanding of OT signaling in the brain which may lead to the development of more targeted interventions for various neuropsychiatric conditions.

Regarding the impact of maternal stress on BDNF, most research has focused on offspring BDNF (Branchi et al., 2013; Liu et al., 2000; Unternaehrer et al., 2015) rather than on maternal BDNF implications. Moreover, we measured individual protein levels of proBDNF and mBDNF, the latter being a cleavage product of proBDNF and, to the best of our knowledge, only one study has distinguished different form of BDNF in response to OT treatment so far (Bukatova et al., 2023), and not in the mother. There is also limited evidence on the effects of probiotics on BDNF levels, with exceptions such as the reduction of circulating BDNF by *Lactobacillus reuteri DSM 17938*—a strain closely related to ATCC PTA-6475—in adults with constipation disorders (Riezzo et al., 2019). Therefore, our study into the maternal BDNF response to stress and *L. reuteri* treatment, is to our knowledge, pioneering.

In this study, we found that both BDNF isoforms were increased in stressed dams and this increase was reversed by *L. reuteri*, with no effect on control of unstressed dams. Notably, proBDNF expression in stressed rats was about 2-fold higher than in controls, while the increase in mBDNF was approximately 1.3-fold. ProBDNF and mBDNF have opposite profiles, with proBDNF being apoptotic and negatively regulating synaptic plasticity, whereas mBDNF enhances synaptic potentiation (Foltran and Diaz, 2016).

Antidepressant treatments promote the cleavage of proBDNF, thereby increasing mBDNF (Lin, 2015). Higher proBDNF levels could be a potential mechanism underlying cognitive deficits in schizophrenia (Carlino et al., 2011). Consequently, we observed that *L. reuteri* overcorrected the imbalance in the proBDNF/mBDNF ratio induced by gestational stress, bringing it back to less than

1-fold in *L. reuteri*-stressed dams compared to the control unstressed water-treated group. Thus, it is likely that *L. reuteri* could act on the conversion of the precursor form to mature BDNF.

This is supported by previous observations suggesting that oxytocin affects BDNF isoforms in the male rat hippocampus during early stages of development, by delaying changes in mBDNF signal with respect to proBDNF, which occur earlier (Bukatova et al., 2023). Moreover, given the apoptotic action of proBDNF and evidence for an inhibitory effect driven by OT on CORT-induced apoptosis in primary hippocampal neurons (Latt et al., 2018), we could speculate about a similar (apoptotic) effect of maternal stress in the hypothalamus which is restored by *postpartum L. reuteri.* Notably, we observed that proBDNF correlated with impaired maternal care and maternal reactivity induced by gestational stress, while mBDNF correlated only with maternal reactivity to pups' separation. Stressed dams exhibiting poor maternal behavior showed decreased OT and increased CORT levels, which both exhibited strong association with mBDNF levels, and in both plasma and brain for OT, while proBDNF was only associated with plasma OT. Therefore, mBDNF showed a more complete association with the CORT/OT balance. Importantly, our findings align with previous human reports of a similar negative correlation between OT and BDNF in the serum of adult subjects (Marazziti et al., 2023). Again, for the first time, we showed that supplementation with *L. reuteri* ATCC PTA 6475, which improved maternal care reduced hypothalamic BDNF protein expression and CORT plasma levels, concurrently with an increase in OT levels. Altogether the specific reduction of BDNF, particularly of proBDNF, in stressed mothers treated with *L. reuteri*, along with increased OT levels, elucidates the mechanism of action of *L. reuteri*. Altogether, our findings suggest that *L. reuteri*, through its oxytocinergic effect on BDNF and CORT levels and its action on the balance of OT, BDNF, and CORT, could be a potential therapeutic avenue for treating psychiatric diseases such as *postpartum* depression (PPD) during the critical lactation period. Although this needs to be proven, we speculate that the oxytocinergic action of *L. reuteri* in stressed dams might occur through the enhancement of pCREB expression. This mechanism has been previously identified in the hippocampus of adult female mice exposed to chronic glucocorticoids following oxytocin administration (Mori et al., 2022), and through the increased phosphorylation of TrkB as shown by rapid transactivation of TrkB by oxytocin in cortical slices of 2-week-old mice (Mitre et al., 2022).

Finally, we emphasize the importance of conducting our molecular investigation in the hypothalamus. This brain region, besides its role in maternal behavior, regulates changes in weight, response to stress, and sociability—factors impacted by depression pathology (Autry, 2022). Thus, our findings in the hypothalamus support continued molecular investigation into PPD.

In conclusion, our investigation in a preclinical model of epigenetic programming provides groundbreaking evidence of the efficacy of *L. reuteri* ATCC PTA 6475 as an oxytocinergic agent able to revert maternal stress and enhance caregiving behaviors, which can be translated to PPD. These

findings present novel strategies for alleviating perinatal psychological burdens in humans and offer potential avenues for translating these insights into actionable clinical interventions aimed at supporting maternal mental health and fostering a stronger mother-infant bond. Treating new mothers affected by PPD with a probiotic, rather than a classical antidepressant, could be very helpful for both the mother and newborn.

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**REFERENCES**

1) Angelucci, L., Patacchioli, F.R., Scaccianoce, S., Di Sciullo, A., Cardillo, A., Maccari, S., 1985. A model for later-life effects of perinatal drug exposure: maternal hormone mediation. Neurobehav. Toxicol. Teratol. 7, 511–517.
2) Asherin, R.M., Everhart, K.D., Stophaeros, S.L., Vogeli, J.M., Fowler, J., Phiel, C.J., Kaplan, P.S., 2020. Associations between maternal depression and mother and infant oxytocin receptor gene (OXTR_rs53576) polymorphisms. Dev. Psychobiol. 62, 496–504. https://doi.org/10.1002/dev.21938
3) Autry, A.E., 2022. Function of brain-derived neurotrophic factor in the hypothalamus: Implications for depression pathology. Front. Mol. Neurosci. 15. https://doi.org/10.3389/fnmol.2022.1028223
4) Barbazanges, A., Piazza, P.V., Moal, M.L., Maccari, S., 1996. Maternal Glucocorticoid

Secretion Mediates Long-Term Effects of Prenatal Stress. J. Neurosci. 16, 3943–3949. https://doi.org/10.1523/JNEUROSCI.16-12-03943.1996

5) Barengolts, E., Green, S.J., Eisenberg, Y., Akbar, A., Reddivari, B., Layden, B.T., Dugas, L., Chlipala, G., 2018. Gut microbiota varies by opioid use, circulating leptin and oxytocin in African American men with diabetes and high burden of chronic disease. PloS One 13, e0194171. https://doi.org/10.1371/journal.pone.0194171
6) Bell, A.F., Carter, C.S., Steer, C.D., Golding, J., Davis, J.M., Steffen, A.D., Rubin, L.H., Lillard, T.S., Gregory, S.P., Harris, J.C., Connelly, J.J., 2015. Interaction between oxytocin receptor DNA methylation and genotype is associated with risk of postpartum depression in women without depression in pregnancy. Front. Genet. 6, 243. https://doi.org/10.3389/fgene.2015.00243
7) Branchi, I., Curley, J.P., D'Andrea, I., Cirulli, F., Champagne, F.A., Alleva, E., 2013. Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology 38, 522–532. https://doi.org/10.1016/j.psyneuen.2012.07.010
8) Buffington, S.A., Viana Di Prisco, G., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli, M., 2016. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell 165, 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001
9) Bukatova, S., Reichova, A., Bacova, Z., Bakos, J., 2023. Neonatal oxytocin treatment alters levels of precursor and mature BDNF forms and modifies the expression of neuronal markers in the male rat hippocampus. Neuropeptides 102, 102384. https://doi.org/10.1016/j.npep.2023.102384
10) Carlino, D., Leone, E., Di Cola, F., Baj, G., Marin, R., Dinelli, G., Tongiorgi, E., De Vanna, M., 2011. Low serum truncated-BDNF isoform correlates with higher cognitive impairment in schizophrenia. J. Psychiatr. Res. 45, 273–279. https://doi.org/10.1016/j.jpsychires.2010.06.012
11) Cataldo, I., Azhari, A., Lepri, B., Esposito, G., 2018. Oxytocin receptors (OXTR) and early parental care: An interaction that modulates psychiatric disorders. Res. Dev. Disabil. 82, 27–38. https://doi.org/10.1016/j.ridd.2017.10.007
12) Champagne, F., Diorio, J., Sharma, S., Meaney, M.J., 2001. Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proc. Natl. Acad. Sci. U. S. A. 98, 12736–12741. https://doi.org/10.1073/pnas.221224598
13) Champagne, F.A., Meaney, M.J., 2006. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. Biol. Psychiatry 59, 1227–1235. https://doi.org/10.1016/j.biopsych.2005.10.016
14) Codagnone, M.G., Spichak, S., O'Mahony, S.M., O'Leary, O.F., Clarke, G., Stanton, C.,

Dinan, T.G., Cryan, J.F., 2019. Programming Bugs: Microbiota and the Developmental Origins of Brain Health and Disease. Biol. Psychiatry 85, 150–163. https://doi.org/10.1016/j.biopsych.2018.06.014

15) Crovesy, L., Ostrowski, M., Ferreira, D.M.T.P., Rosado, E.L., Soares-Mota, M., 2017. Effect of Lactobacillus on body weight and body fat in overweight subjects: a systematic review of randomized controlled clinical trials. Int. J. Obes. 2005 41, 1607–1614. https://doi.org/10.1038/ijo.2017.161
16) Danhof, H.A., Lee, J., Thapa, A., Britton, R.A., Di Rienzi, S.C., 2023. Microbial stimulation of oxytocin release from the intestinal epithelium via secretin signaling. BioRxiv Prepr. Serv. Biol. 2023.03.09.531917. https://doi.org/10.1101/2023.03.09.531917
17) Dinan, T.G., Stanton, C., Cryan, J.F., 2013. Psychobiotics: A Novel Class of Psychotropic. Biol. Psychiatry, Novel Therapeutics for Depression 74, 720–726. https://doi.org/10.1016/j.biopsych.2013.05.001
18) Duffy, K.A., Sammel, M.D., Johnson, R.L., Morrison, K.E., Bale, T.L., Epperson, C.N., 2024. Sex differences in stress-induced cortisol response among infants of mothers exposed to childhood adversity. Biol. Psychiatry S0006-3223(24)01350–7. https://doi.org/10.1016/j.biopsych.2024.05.015
19) Feldman, R., Granat, A., Pariente, C., Kanety, H., Kuint, J., Gilboa-Schechtman, E., 2009. Maternal depression and anxiety across the postpartum year and infant social engagement, fear regulation, and stress reactivity. J. Am. Acad. Child Adolesc. Psychiatry 48, 919–927. https://doi.org/10.1097/CHI.0b013e3181b21651
20) Foltran, R.B., Diaz, S.L., 2016. BDNF isoforms: a round trip ticket between neurogenesis and serotonin? J. Neurochem. 138, 204–221. https://doi.org/10.1111/jnc.13658
21) Gareau, M.G., Jury, J., MacQueen, G., Sherman, P.M., Perdue, M.H., 2007. Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 56, 1522–1528. https://doi.org/10.1136/gut.2006.117176
22) Gatta, E., Mairesse, J., Deruyter, L., Marrocco, J., Van Camp, G., Bouwalerh, H., Lo Guidice, J.-M., Morley-Fletcher, S., Nicoletti, F., Maccari, S., 2018. Reduced maternal behavior caused by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance. NeuroToxicology 66, 138–149. https://doi.org/10.1016/j.neuro.2018.04.005
23) Havranek, T., Zatkova, M., Lestanova, Z., Bacova, Z., Mravec, B., Hodosy, J., Strbak, V., Bakos, J., 2015. Intracerebroventricular Oxytocin Administration in Rats Enhances Object Recognition and Increases Expression of Neurotrophins, Microtubule-Associated Protein 2, and Synapsin I. J. Neurosci. Res. 93, 893–901. https://doi.org/10.1002/jnr.23559
24) Herring, S.J., Rich-Edwards, J.W., Oken, E., Rifas-Shiman, S.L., Kleinman, K.P., Gillman, M.W., 2008. Association of Postpartum Depression With Weight Retention 1 Year After

Childbirth. Obes. Silver Spring Md 16, 1296–1301. https://doi.org/10.1038/oby.2008.71

25) Kingsbury, M.A., Bilbo, S.D., 2019. The inflammatory event of birth: How oxytocin signaling may guide the development of the brain and gastrointestinal system. Front. Neuroendocrinol. 55, 100794. https://doi.org/10.1016/j.yfrne.2019.100794

26) Latt, H.M., Matsushita, H., Morino, M., Koga, Y., Michiue, H., Nishiki, T., Tomizawa, K., Matsui, H., 2018. Oxytocin Inhibits Corticosterone-induced Apoptosis in Primary Hippocampal Neurons. Neuroscience 379, 383–389. https://doi.org/10.1016/j.neuroscience.2018.03.025

27) Lee, Y.M., Mu, A., Wallace, M., Gengatharan, J.M., Furst, A.J., Bode, L., Metallo, C.M., Ayres, J.S., 2021. Microbiota control of maternal behavior regulates early postnatal growth of offspring. Sci. Adv. 7, eabe6563. https://doi.org/10.1126/sciadv.abe6563

28) Lin, P.-Y., 2015. Regulation of proteolytic cleavage of brain-derived neurotrophic factor precursor by antidepressants in human neuroblastoma cells. Neuropsychiatr. Dis. Treat. 11, 2529–2532. https://doi.org/10.2147/NDT.S87743

29) Liu, D., Diorio, J., Day, J.C., Francis, D.D., Meaney, M.J., 2000. Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat. Neurosci. 3, 799–806. https://doi.org/10.1038/77702

30) Liu, Y., Zhong, X., Lin, S., Xu, H., Liang, X., Wang, Y., Xu, J., Wang, K., Guo, X., Wang, J., Yu, M., Li, C., Xie, C., 2022. Limosilactobacillus reuteri and caffeoylquinic acid synergistically promote adipose browning and ameliorate obesity-associated disorders. Microbiome 10, 226. https://doi.org/10.1186/s40168-022-01430-9

31) Lopes, N.A., Ambeskovic, M., King, S.E., Faraji, J., Soltanpour, N., Falkenberg, E.A., Scheidl, T., Patel, M., Fang, X., Metz, G.A.S., Olson, D.M., 2023. Environmental Enrichment Promotes Transgenerational Programming of Uterine Inflammatory and Stress Markers Comparable to Gestational Chronic Variable Stress. Int. J. Mol. Sci. 24, 3734. https://doi.org/10.3390/ijms24043734

32) Maccari, S., Krugers, H.J., Morley-Fletcher, S., Szyf, M., Brunton, P.J., 2014. The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. J. Neuroendocrinol. 26, 707–723. https://doi.org/10.1111/jne.12175

33) Maccari, S., Piazza, P.V., Kabbaj, M., Barbazanges, A., Simon, H., Le Moal, M., 1995. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J. Neurosci. Off. J. Soc. Neurosci. 15, 110–116.

34) Maccari, S., Polese, D., Reynaert, M.-L., Amici, T., Morley-Fletcher, S., Fagioli, F., 2017. Early-life experiences and the development of adult diseases with a focus on mental illness: The Human Birth Theory. Neuroscience 342, 232–251. https://doi.org/10.1016/j.neuroscience.2016.05.042

35) Marazziti, D., Baroni, S., Mucci, F., Palego, L., Arone, A., Betti, L., Palermo, S., Giannaccini,

G., Carbone, M.G., Dell'Osso, L., 2023. Relationship between BDNF and oxytocin. Compr. Psychoneuroendocrinology 16, 100207. https://doi.org/10.1016/j.cpnec.2023.100207

36) Marrocco, J., Mairesse, J., Ngomba, R.T., Silletti, V., Van Camp, G., Bouwalerh, H., Summa, M., Pittaluga, A., Nicoletti, F., Maccari, S., Morley-Fletcher, S., 2012. Anxiety-Like Behavior of Prenatally Stressed Rats Is Associated with a Selective Reduction of Glutamate Release in the Ventral Hippocampus. J. Neurosci. 32, 17143–17154. https://doi.org/10.1523/JNEUROSCI.1040-12.2012
37) Maynard, K.R., Hobbs, J.W., Phan, B.N., Gupta, A., Rajpurohit, S., Williams, C., Rajpurohit, A., Shin, J.H., Jaffe, A.E., Martinowich, K., 2018. BDNF-TrkB signaling in oxytocin neurons contributes to maternal behavior. eLife 7, e33676. https://doi.org/10.7554/eLife.33676
38) Meaney, M.J., 2010. Epigenetics and the biological definition of gene x environment interactions. Child Dev. 81, 41–79. https://doi.org/10.1111/j.1467-8624.2009.01381.x
39) Mitre, M., Saadipour, K., Williams, K., Khatri, L., Froemke, R.C., Chao, M.V., 2022. Transactivation of TrkB Receptors by Oxytocin and Its G Protein-Coupled Receptor. Front. Mol. Neurosci. 15, 891537. https://doi.org/10.3389/fnmol.2022.891537
40) Mori, M., Shizunaga, H., Harada, H., Tajiri, Y., Murata, Y., Terada, K., Ohe, K., Enjoji, M., 2022. Oxytocin treatment improves dexamethasone-induced depression-like symptoms associated with enhancement of hippocampal CREB-BDNF signaling in female mice. Neuropsychopharmacol. Rep. 42, 356–361. https://doi.org/10.1002/npr2.12271
41) Morley-Fletcher, S., Gaetano, A., Gao, V., Gatta, E., Van Camp, G., Bouwalerh, H., Thomas, P., Nicoletti, F., Maccari, S., 2024. Postpartum Oxytocin Treatment via the Mother Reprograms Long-Term Behavioral Disorders Induced by Early Life Stress on the Plasma and Brain Metabolome in the Rat. Int. J. Mol. Sci. 25, 3014. https://doi.org/10.3390/ijms25053014
42) Morley-Fletcher, S., Zuena, A.R., Mairesse, J., Gatta, E., Van Camp, G., Bouwalerh, H., Riozzi, B., Battaglia, G., Pittaluga, A., Olivero, G., Mocaer, E., Bretin, S., Nicoletti, F., Maccari, S., 2018. The reduction in glutamate release is predictive of cognitive and emotional alterations that are corrected by the positive modulator of AMPA receptors S 47445 in perinatal stressed rats. Neuropharmacology 135, 284–296. https://doi.org/10.1016/j.neuropharm.2018.03.018
43) O'Mahony, S.M., McVey Neufeld, K.-A., Waworuntu, R.V., Pusceddu, M.M., Manurung, S., Murphy, K., Strain, C., Laguna, M.C., Peterson, V.L., Stanton, C., Berg, B.M., Dinan, T.G., Cryan, J.F., 2020. The enduring effects of early-life stress on the microbiota-gut-brain axis are buffered by dietary supplementation with milk fat globule membrane and a prebiotic blend. Eur. J. Neurosci. 51, 1042–1058. https://doi.org/10.1111/ejn.14514
44) Payne, J.L., Maguire, J., 2019. Pathophysiological Mechanisms Implicated in Postpartum Depression. Front. Neuroendocrinol. 52, 165–180. https://doi.org/10.1016/j.yfrne.2018.12.001
45) Poutahidis, T., Kearney, S.M., Levkovich, T., Qi, P., Varian, B.J., Lakritz, J.R., Ibrahim, Y.M.,

Chatzigiagkos, A., Alm, E.J., Erdman, S.E., 2013. Microbial Symbionts Accelerate Wound Healing via the Neuropeptide Hormone Oxytocin. PLoS ONE 8, e78898. https://doi.org/10.1371/journal.pone.0078898

46) Rathi, A., Khapre, S., Chavada, J., Gupta, S., Singla, T., 2022. Postpartum Depression and Its Biological Biomarkers. Cureus 14. https://doi.org/10.7759/cureus.31124

47) Riezzo, G., Chimienti, G., Orlando, A., D'Attoma, B., Clemente, C., Russo, F., 2019. Effects of long-term administration of Lactobacillus reuteri DSM-17938 on circulating levels of 5-HT and BDNF in adults with functional constipation. Benef. Microbes 10, 137–147. https://doi.org/10.3920/BM2018.0050

48) Righetti-Veltema, M., Bousquet, A., Manzano, J., 2003. Impact of postpartum depressive symptoms on mother and her 18-month-old infant. Eur. Child Adolesc. Psychiatry 12, 75–83. https://doi.org/10.1007/s00787-003-0311-9

49) Robinson, C., Schumann, R., Zhang, P., Young, R.C., 2003. Oxytocin-induced desensitization of the oxytocin receptor. Am. J. Obstet. Gynecol. 188, 497–502. https://doi.org/10.1067/mob.2003.22

50) Roth, T.L., Lubin, F.D., Funk, A.J., Sweatt, J.D., 2009. LASTING EPIGENETIC INFLUENCE OF EARLY-LIFE ADVERSITY ON THE BDNF GENE. Biol. Psychiatry 65, 760–769. https://doi.org/10.1016/j.biopsych.2008.11.028

51) Sanson, A., Bosch, O.J., 2022. Dysfunctions of brain oxytocin signaling: Implications for poor mothering. Neuropharmacology 211, 109049. https://doi.org/10.1016/j.neuropharm.2022.109049

52) Sgritta, M., Dooling, S.W., Buffington, S.A., Momin, E.N., Francis, M.B., Britton, R.A., Costa-Mattioli, M., 2019. Mechanisms underlying microbial-mediated changes in social behavior in mouse models of Autism Spectrum Disorder. Neuron 101, 246-259.e6. https://doi.org/10.1016/j.neuron.2018.11.018

53) Singh, S., Fereshetyan, K., Shorter, S., Paliokha, R., Dremencov, E., Yenkoyan, K., Ovsepian, S.V., 2023. Brain-derived neurotrophic factor (BDNF) in perinatal depression: Side show or pivotal factor? Drug Discov. Today 28, 103467. https://doi.org/10.1016/j.drudis.2022.103467

54) Thul, T.A., Corwin, E.J., Carlson, N.S., Brennan, P.A., Young, L.J., 2020. Oxytocin and postpartum depression: A systematic review. Psychoneuroendocrinology 120, 104793. https://doi.org/10.1016/j.psyneuen.2020.104793

55) Toepfer, P., O'Donnell, K.J., Entringer, S., Garg, E., Heim, C.M., Lin, D.T.S., MacIsaac, J.L., Kobor, M.S., Meaney, M.J., Provençal, N., Binder, E.B., Wadhwa, P.D., Buss, C., 2019. Dynamic DNA methylation changes in the maternal oxytocin gene locus (OXT) during pregnancy predict postpartum maternal intrusiveness. Psychoneuroendocrinology 103, 156–162. https://doi.org/10.1016/j.psyneuen.2019.01.013

56) Triana-Del Rio, R., Ranade, S., Guardado, J., LeDoux, J., Klann, E., Shrestha, P., 2022. The

modulation of emotional and social behaviors by oxytocin signaling in limbic network. Front. Mol. Neurosci. 15, 1002846. https://doi.org/10.3389/fnmol.2022.1002846

57) Turecki, G., Meaney, M.J., 2016. Effects of the Social Environment and Stress on Glucocorticoid Receptor Gene Methylation: A Systematic Review. Biol. Psychiatry 79, 87–96. https://doi.org/10.1016/j.biopsych.2014.11.022

58) Unternaehrer, E., Meyer, A.H., Burkhardt, S.C.A., Dempster, E., Staehli, S., Theill, N., Lieb, R., Meinlschmidt, G., 2015. Childhood maternal care is associated with DNA methylation of the genes for brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OXTR) in peripheral blood cells in adult men and women. Stress Amst. Neth. 18, 451–461. https://doi.org/10.3109/10253890.2015.1038992

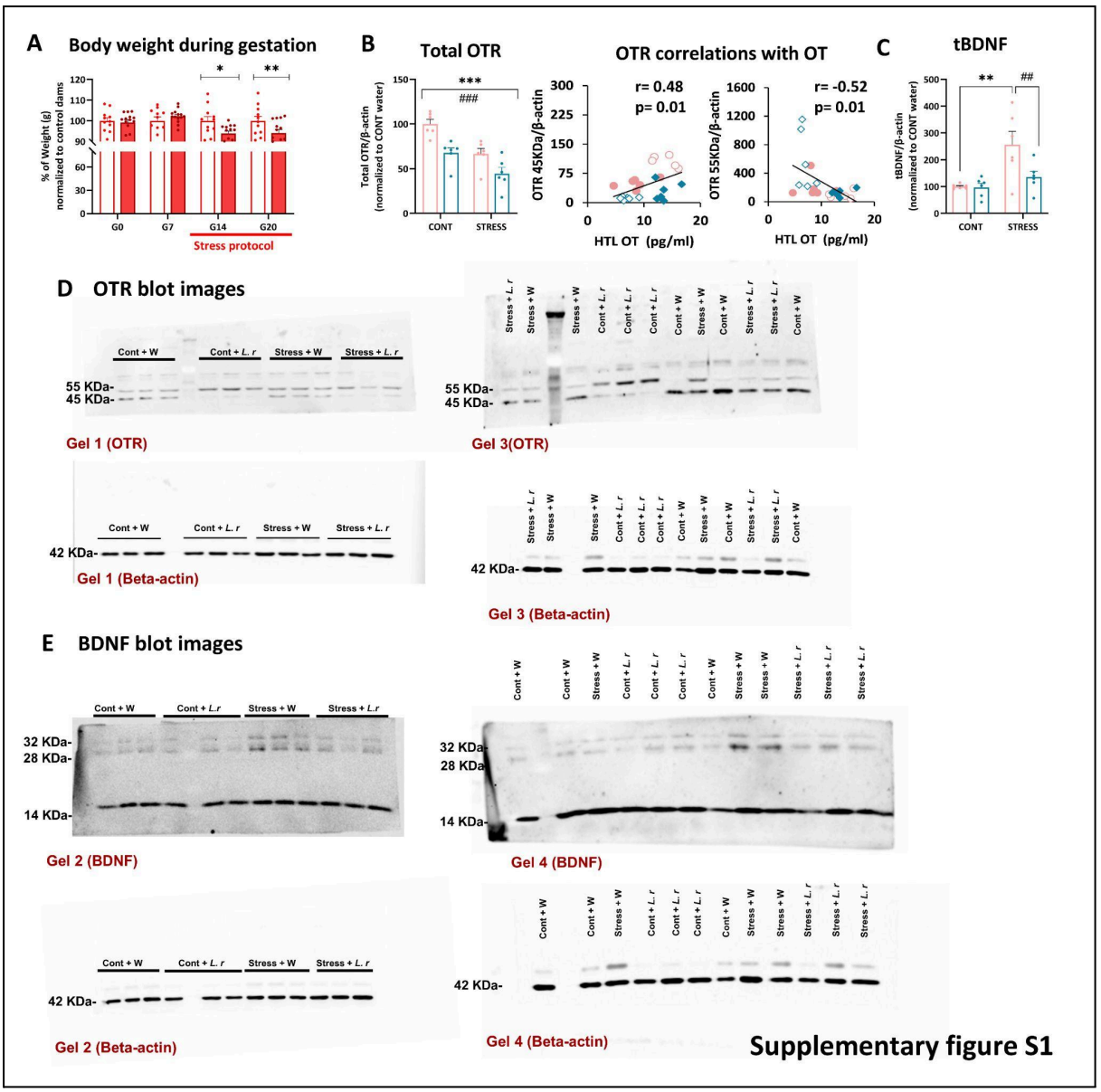
59) Varian, B.J., Poutahidis, T., DiBenedictis, B.T., Levkovich, T., Ibrahim, Y., Didyk, E., Shikhman, L., Cheung, H.K., Hardas, A., Ricciardi, C.E., Kolandaivelu, K., Veenema, A.H., Alm, E.J., Erdman, S.E., 2017. Microbial lysate upregulates host oxytocin. Brain. Behav. Immun. 61, 36–49. https://doi.org/10.1016/j.bbi.2016.11.002

60) Vicariotto, F., Malfa, P., Torricelli, M., Lungaro, L., Caio, G., De Leo, V., 2023. Beneficial Effects of Limosilactobacillus reuteri PBS072 and Bifidobacterium breve BB077 on Mood Imbalance, Self-Confidence, and Breastfeeding in Women during the First Trimester Postpartum. Nutrients 15, 3513. https://doi.org/10.3390/nu15163513

61) Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854. https://doi.org/10.1038/nn1276

62) Zhu, J., Jin, J., Tang, J., 2023. Oxytocin and Women Postpartum Depression: A Systematic Review of Randomized Controlled Trials. Neuropsychiatr. Dis. Treat. 19, 939–947. https://doi.org/10.2147/NDT.S393499

63) Zoubovsky, S.P., Hoseus, S., Tumukuntala, S., Schulkin, J.O., Williams, M.T., Vorhees, C.V., Muglia, L.J., 2020. Chronic psychosocial stress during pregnancy affects maternal behavior and neuroendocrine function and modulates hypothalamic CRH and nuclear steroid receptor expression. Transl. Psychiatry 10, 1–13. https://doi.org/10.1038/s41398-020-0704-2




**Supplementary Figure 1.** displays body weight during gestation (**A**). Total OTR protein quantification and the correlations of its different isoforms with OT in the HTL (**B**). Truncated BDNF (tBDNF) protein quantification (**C**). The Western blot membranes from gels used for BDNF and OTR, which were utilized for quantification purposes. The membranes captured in the images were not sectioned (**D-E**); rather, only the membranes themselves were horizontally cut around 40 kDa. The first segments were allocated for OTR (45KDa and 55 KDa) and beta-actin (42 KDa) blotting. Conversely, the second segments of the membranes were dedicated to BDNF blotting. Each target protein was run on two separate gels, and for each group, samples from six animals were included. All values are means ± S.E.M. (n=6 rats/group for WB and n=11-14 rat/group for gestational weight), * $p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ for CONT *vs.* STRESS, and ## $p < 0.01$; ### $p < 0.001$ for water *vs. L. reuteri* corresponding group.

**Supplementary Table 1.** Statistical analysis of correlation data among maternal behaviors and biochemical correlates (OTR and BDNF protein expression) in the hypothalamus and stress anti-stress balance in the plasma. significance in bold.

| **Interactions** | STRESS and CONT (water and *L. reuteri*) | |
|---|---|---|
| | r-value | p-value |
| **Maternal behaviors** | | |
| **Licking latency x Maternal care** | **-0.51** | **0.01 **** |
| **with Maternal care** | | |
| CORT | -0.33 | 0.12 |
| **OT (plasma)** | **0.46** | **0.026*** |
| OT (HTL) | -0.27 | 0.19 |
| OTR (45 kDa) | 0.04 | 0.82 |
| OTR (55kDa-glycosylated) | -0.03 | 0.89 |
| OTR total | 0.05 | 0.78 |
| **ProBDNF** | **-0.47** | **0.02*** |
| tBDNF | -0.25 | 0.24 |
| mBDNF | -0.33 | 0.11 |
| **with Licking latency following pups' reunion (separation test)** | | |
| **CORT** | **0.48** | **0.01**** |
| **OT (plasma)** | **-0.54** | **0.007**** |
| OT (HTL) | -0.16 | 0.45 |
| OTR (45kDa) | 0.17 | 0.43 |
| OTR (55kDa) | 0.15 | 0.47 |
| OTR total | 0.23 | 0.28 |
| **ProBDNF** | **0.65** | **0.007**** |
| tBDNF | 0.29 | 0.17 |
| **mBDNF** | **0.62** | **0.001***** |

**Supplementary Table 2.** Statistical analysis of correlation data among the stress/anti-stress balance and biochemical correlates (OTR and BDNF protein expression) in the hypothalamus, significance in bold.

| **Interactions** | **STRESS and CONT (water and *L. reuteri*)** | |
|---|---|---|
| | r-value | p-value |
| **Stress/anti-stress balance in plasma** | | |
| **OT x CORT in plasma** | **-0.51** | **0.01**** |
| **with CORT** | | |
| OT (HTL) | -0.28 | 0.19 |
| OTR (45kDa) | 0.04 | 0.82 |
| OTR (55kDa-glycosylated) | -0.14 | 0.5 |
| OTR total | 0.09 | 0.66 |
| proBDNF | 0.29 | 0.16 |
| **tBDNF** | **0.39** | ***0.06*** |
| **mBDNF** | **0.57** | **0.003**** |
| **with OT (plasma)** | | |
| OT (HTL) | 0.35 | 0.10 |
| OTR (45kDa) | -0.25 | 0.24 |
| OTR (55kDa-glycosylated) | -0.07 | 0.74 |
| OTR total | 0.3 | 0.16 |
| **proBDNF** | **-0.51** | **0.01*** |
| tBDNf | -0.32 | 0.12 |
| **mBDNF** | **-0.64** | **0.0009***** |
| **with OT (hypothalamus)** | | |
| **OTR (45kDa)** | **0.48** | **0.01*** |
| **OTR (55kDa-glycosylated)** | **-0.52** | **0.01*** |
| OTR total | 0.07 | 0.72 |
| proBDNF | -0.32 | 0.12 |
| tBDNF | -0.24 | 0.26 |
| **mBDNF** | **-0.53** | **0.007**** |

### B. Evaluating the Impact of *Limosilactobacillus reuteri* on Reversing PRS Deficits in Offspring *(Article n° 4 in preparation)*

From the offspring's perspective, there is accumulating evidence in the literature indicating that maternal behavior can imprint lasting behavioral and molecular changes in the offspring, a phenomenon known as programming (Bale et al., 2010; Jašarević et al., 2014). This process suggests that early-life experiences, including maternal stress, can significantly influence offspring development and health outcomes across their lifespan. Previous research has highlighted the potential of probiotics, such as *Limosilactobacillus reuteri*, to modulate HPA axis activity (Varian et al., 2017) and stress-related behaviors in both rodents and humans (H. J. Lee et al., 2021; Park et al., 2021), serving as an anti-stress to mitigate related disorders. Building on this foundation, we extend the previous findings to examine offspring outcomes. The primary aim of this section investigates whether probiotic supplementation administered to stressed dams during the first *postpartum* week could have a persisting effect on the offspring and alleviate PRS-related deficits and restore them. We addressed particularly the stress-anti-stress balance, focusing on behavioral parameters related to risk-taking behavior in the EPM and the molecular marker of stress  plasma corticosterone levels.

By investigating these aspects, our study seeks to elucidate not only the programming effects of maternal distress on offspring, as documented in existing literature of the perinatal stress model (Gatta et al., 2018, 2018; Morley-Fletcher et al., 2024), but also the persistence of the corrective effects of probiotic interventions administered to mothers. This research hopes to identify novel therapeutic avenues aimed at mitigating the long-term consequences of maternal stress on offspring development.

## Article n° 4 in preparation

*Article n° 4 in preparation*

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# Investigation of the transmission of the beneficial effect of *postpartum* *Limosilactobacillus reuteri* treatment in stressed dams on the PRS male offspring: OTR/BDNF and Sialylation interplay

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## CONTEXT

Gastrointestinal issues and changes in the composition of the microbiome often associated with chronic inflammatory diseases, metabolic syndrome and neuropathologies (Hoarau et al., 2016). Early-life stress (ELS) in both humans and experimental models contributes to similar pathologies associated with altered microbiota, which co-develops with the brain during early life (Dirven et al., 2017; Maccari et al., 2017). In this context, probiotics, sometimes referred to as psychobiotics (Bermúdez-Humarán et al., 2019), can help restore gut microbiota balance and potentially benefit conditions related to inflammation, immune hyperactivation, and metabolic syndrome linked to stress-endocrine-immune disorders (Dinan et al., 2013). Various molecular factors come into play in maternal behavior programming, a complex process through which maternal experiences during pregnancy influence the long-term health and development of offspring. These include maternal glucocorticoids and oxytocin (OT). For instance, glucocorticoids impact neuroplasticity (Pesarico et al., 2019), induce inflammation (Tret'yakova et al., 2023; Vanbesien-Mailliot et al., 2007) while OT plays a crucial role in maternal bonding and social attachment, it also exerts anti-stress effects (Carter et al., 2020) and positively regulates systemic immune activity (Amini-Khoei et al., 2017; Friuli et al., 2021). Adverse environmental challenges during perinatal life disrupt the balance between glucocorticoids and OT, leading to disturbed phenotypes in the offspring of exposed mothers. The preclinical model of perinatal stress (PRS) in rats, showed that the offspring that remain with their stressed biological mother until weaning continue to receive defective maternal care, resulting in permanent alterations in HPA activity and regulation, a systemic proinflammatory profile with higher

levels of circulating IL-6, IFN-γ, and CD8+ cells (Vanbesien-Mailliot et al., 2007; Verhaeghe et al., 2021), as well as reduced hippocampal neurogenesis (Morley-Fletcher et al., 2011). Inflammatory responses, which are intensified by stress, also influence sialylation. This occurs through elevated levels of circulating proinflammatory cytokines, such as IL-6 and IL-8, which decrease the expression of the sialyltransferase ST6GAL1 (Krick et al., 2021). Another sialyltransferase such as ST8SIA4 is also involved in the polysialylation of the Neural Cell Adhesion Molecule (PSA-NCAM) which is reduced in the PRS rat model (Morley-Fletcher et al., 2011). Notably, treatment with the OTR agonist carbetocin, administered to the descendants (Mairesse et al., 2015) or to the lactating mother shortly in *postpartum* (Gatta et al., 2018), can reverse the programming of the PRS phenotype at both the behavioral and molecular level by increasing maternal behavior (Gatta et al., 2018; Morley-Fletcher et al., 2024). In this context, a new strategy using OT enhancement is emerging, including probiotic supplementations. Indeed strains, like *Lactobacillus reuteri* have been found to have OT-activating properties, increasing wound healing by upregulating circulating OT levels in adult mice and activating lymphocytes, through increased expression of FoxP3 T-regulatory cells (Poutahidis et al., 2013). We have proven evidence that *L. reuteri* given as a *postpartum* treatment during the first week of lactation to stressed mothers is able to correct the deficit in maternal behavior induced by stress by acting on the stress/anti-stress balance of the mother as well as on the OT/BDNF interplay in the brain. In this ongoing work, we aimed to determine whether upon correction of maternal behavior, *L. reuteri* had the ability to counteract the programming in the PRS adult offspring by focusing on the behavioral and molecular correlates which are characteristic of the PRS phenotype such risk-taking behavior, elevated corticosterone response to stressors and increased BDNF protein expression and we also investigated the protein expression of OTR in the hippocampus. Additionally, we used qPCR to quantify the gene expression of sialyltransferases ST8SIA4 and ST6GAL1, the enzymes involved in the polysialylation of the neural cell adhesion and inflammatory response, respectively.

*This part of the study was conducted with the help of Maria Grazia Ngene Tchokonte (Master 2 student in Biology & health) and Camille Dupont (Master 1 student in Bioinformatics, Omics and Systems Biology Pathway) who performed their internship in the GlycoStress team during 2023-2024. I actively participated in supervising their experiments.*

## MATERIAL AND METHODS

**Ethics, animal experimentations, Risk-taking behavior in the EPM, BDNF and OTR protein expression in the ventral hippocampus:** Were all previously described above in the part of the mothers experiment of the paper ” *Limosilactobacillus reuteri* Supplementation in Lactating Rats Improves Maternal Behavior and Hypothalamic Oxytocin/ Brain-derived neurotrophic factor balance, Offering Insights for *Postpartum* Depression”.

**CORT measurements after novelty stress exposure:** To evaluate HPA axis activity, plasma was collected and measured for CORT levels from tail's blood after 30 minutes exposure to novelty stress as previously mentioned in the first paper of the thesis " Transient *postpartum* activation of oxytocin receptor prevents postnatal intergenerational inheritance of early life stress".

**Quantitative mRNA analysis of ST8SIA4 and ST6GAL1 in the ventral hippocampus:** In adult males, ventral hippocampus were homogenized in RNA-free tubes using a TissueRuptor (QIAGEN) and Tri Reagent®, after that cDNA synthesis was carried out using the First Strand cDNA synthesis kit (#K1641, Thermo Fisher). Gene expression of ST8SIA4, ST6GAL and the housekeeping gene HPRT were quantified with Maxima SYBR Green qPCR Master Mix 2X (#K0251, Thermo Scientific™). The qPCR reaction was carried out using the AriaMx Real-Time PCR system (Agilent Technologies). For graphical representation, the relative mRNA expression is shown as a ratio compared to the CONT water group. The following primers were used: (ST6GAL1) forward: agatgccatgggaactgtgg and reverse**:** agggtggctttcccaaacaa. (ST8SIA4) forward: agaagcacgtggaatgggtt and reverse: tgtgaggacttgcgttggaa. (HPRT) forward: tcccagcgtcgtgattagtg and reverse: tggcctcccatctccttcat.

**Statistics:** In this exploratory study (ongoing), statistical analyses were conducted using Statistica™ software version 14.1.0.8 (StatSoft Inc.). The normality of data distribution was assessed using the Shapiro-Wilk test, and then a Two-ways ANOVA with Newman-Keuls post-hoc (NK) or Planned Comparisons (PC) tests were applied. Independent variables included group* (PRS *vs*. CONT) and treatment# (Water *vs*. *L. reuteri*). A p-value of $< 0.05$ was considered statistically significant. Graphical representations were created using GraphPad Prism version 10.2.3.

**RESULTS**

***L. reuteri* mitigated the reduction of risk-taking behavior in PRS:** The % of time spent in the open arm was reduced by PRS, indicating a reduction of risk-taking behavior when compared to the CONT group **(Figure 1A)**. This profile was fully reversed by *L. reuteri* which increased the % of time in the open arms in PRS rats (n=10 rats/group. *PRS* × *L. reuteri interaction* $F_{(1,36)}=15.752$; $p< 0.001$. *PRS effect* $F_{(1,36)}=15$ 4.337; p=0.044. NK, CONT Water *vs*. PRS Water ***p=0.0008. PRS Water *vs*. PRS *L. reuteri* #p=0.020. CONT Water *vs*. CONT *L.reuteri* #p=0.015). These results are easily visualized in the heatmaps of the EPM of **Figure 1A**.

***L. reuteri* normalized the increased plasmatic CORT in PRS males:** Using ELISA test, we evidenced in the PRS male offspring an increase of CORT level in the plasma after 30 minutes exposures to a novelty stress, which was reduced by the probiotic that was administered in their mothers during the 1st *postpartum* week (*PRS x probiotic effec*t; $F_{(1,21)}=4.44$; */#p=0.04; **Figure 1B**).

**Effect of PRS and/or *L. reuteri* on the expression of OTR in the ventral hippocampus of the offspring:** Western blot quantification did not show any impact of PRS and as well as the probiotic in native OTR expression in the ventral hippocampus (**Figure 1C**), when considering the glycosylated OTR, PRS induced an increase of its expression independently of *L. reuteri* treatment (*PRS effect* $F_{(1,20)}$=5.682, *p*=0.027; PRS water *vs.* CONT water ***p*=0.007, PC) (**Figure 1C**).

**Effect of PRS and/or *L. reuteri* on the protein expression of mBDNF and proBDNF in the ventral hippocampus of male rats:** Independently of *L. reuteri* treatment, the PRS group presented higher levels of proBDNF in the ventral hippocampus (*PRS effect* $F_{(1,20)}$=4.474, *p*=0.047; CONT water *vs.* PRS water **p*=0.044, PC). Indeed, PRS induced a 4-fold increase of the quantity of proBDNF, and the probiotic did not show any effect on proBDNF expression (Figure 1D). mature BDNF showed a tendency to increase by PRS when *L. reuteri* did not have any effect (*p*=0.06, PC) (**Figure 1D**).

**PRS reduced ST8SIA4 and ST6GAL1 expression in the ventral hippocampus:** Gene expression analysis showed that both ST8SIA4 and ST6GAL1 genes were decreased in the PRS group compared to CONT, however the probiotic failed to rescue this reduction (ST8SIA4 n=5-8 rats/group. *PRS* × *L. reuteri interaction* $F_{(1,24)}$=3.973; p=0.057. PC, CONT *vs*. PRS in water group *p=0.018; Water *vs*. *L. reuteri* in control group #p=0.059), (ST6GAL1, n=5-8 rats/group. *PRS effect* $F_{(1,24)}$=5, p=0.034; PC, CONT *vs*. PRS in water group *p=0.028) (**Figure 1E**).

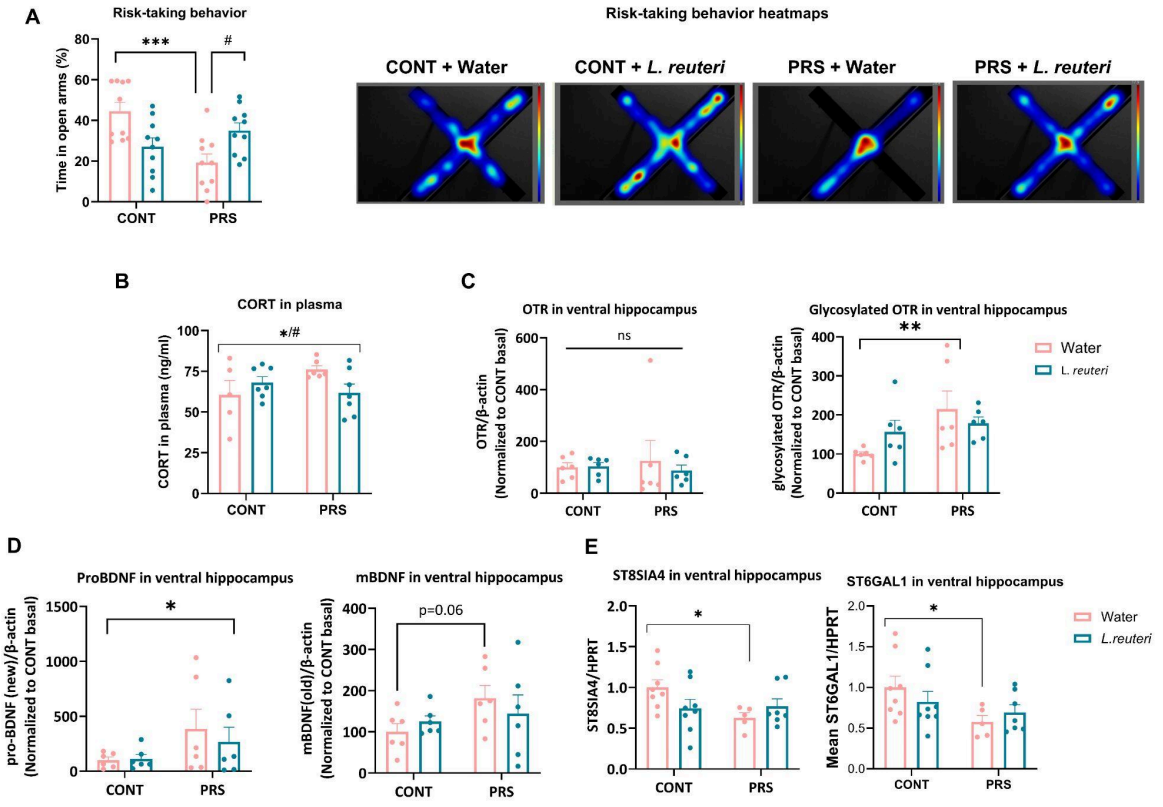


**Figure 1. Impact of PRS on male offspring and the protective effect of *L.reuteri* administered in F0 dams.** **A)** Risk-taking behavior and their heatmaps in the EPM. **B)** CORT levels in the plasma. **C)** In the ventral hippocampus expression of: OTR and glycosylated OTR protein levels. **D)** proBDNF and mBDNF protein levels. **E)** ST8SIA4 and ST6GAL1 mRNA.

## DISCUSSION

This study assessed if *L. reuteri* treatment in stressed dams could reverse PRS deficits in male offspring, focusing on behavioral, endocrine, and neurochemical aspects, especially the OT/BDNF-sialylation interplay. Results showed *L. reuteri* increased risk-taking behavior and reduced CORT levels in PRS adult progeny. The glycosylated oxytocin receptor (OTR) form was most implicated, and PRS increased proBDNF more than mature BDNF. PRS also altered sialyltransferases expression, with *L. reuteri* reversing the behavioral and endocrine deficits while neurochemical deficits in the ventral hippocampus were partially reversed. The choice to investigate in the ventral hippocampus was because the region is responsible for stress response, emotion and fear, compared to the dorsal region that controls memory.

Our positive results concerning risk-taking behavior and reduced CORT levels after exposure to novelty with *L. reuteri* confirm the anti-stress effects of this strain. This observation aligns with previous studies indicating that the HPA axis and stress are central to many psychiatric disorders and that probiotics use can reduce anxiety-like behaviors in animal models, with implications for human mental health (Cryan and Dinan, 2012). For instance, an investigation reported reduced CORT levels in mice treated with *L. reuteri*, providing evidence of the probiotic's anti-stress capacities (Varian et al., 2017). Similarly, Patterson and colleagues found that specific *lactobacilli* strains positively influenced metabolism and reduced depressive-like behavior in mice, suggesting potential therapeutic applications for probiotics in metabolic and mood-related disorders (Patterson et al., 2019). The added value of the current study is the highlight of the programming capacity of *L. reuteri* since the probiotic was administered to the mothers rather than directly to the offspring.

Regarding the anti-stress parameter, we investigated the oxytocin receptor (OTR) and its glycosylated form. While the role of glycosylation on this receptor's function remains unclear, our findings showed that the native OTR was unaffected by both PRS and the probiotic. In contrast, the glycosylated OTR exhibited higher levels in response to PRS. This aligns with previous studies demonstrating increased OTR gene expression in the hippocampus, though those studies did not distinguish between OTR isoforms (Gatta et al., 2018). Notably, our findings differ from the reported reduction in the 60kDa glycosylated OTR form due to PRS (Gatta et al., 2018). Early investigations revealed that mutations in the N-glycosylation sites of OTR do not affect their dissociation constant (Kd) or affinities for

oxytocin-related ligands, suggesting that full glycosylation of OTR is not essential for its activity (Kimura et al., 1997).

In the synaptosomal extracts, BDNF regulation was evident, showing a higher increase in proBDNF and less significant changes in mBDNF in the ventral hippocampus. Although the probiotic failed to rescue proBDNF levels, this highlights PRS's potential to favor apoptosis in the synaptosomal fraction of the hippocampus. ProBDNF is more implicated in apoptosis and synaptic depression compared to its mature form, as it binds to different receptors and has distinct biological activities (Yang et al., 2014).

The PRS model in the ventral hippocampus was shown to reduce neurogenesis and neuroplasticity through the reduction of PSA-NCAM, a marker of neuroplasticity that is polysialylated. Polysialylation is controlled by the enzymatic activity of sialyltransferases. Our study investigated if ST8SIA4 was impacted by PRS and found that PRS reduced its expression in the ventral hippocampus, which corroborated studies that showed that acute stress reduced the quantity and quality of polySia in the olfactory bulb and prefrontal cortex, even with short-term exposure to stress (Abe et al., 2019). Chronically stressed rats displayed reduced hippocampal neural cell adhesion molecules (Sandi et al., 2001). Moreover, glycosylation, specifically polysialylation, is implicated in both plasticity and inflammation. ST6GAL directly regulates IL-6 expression, a hallmark of proinflammatory profiles, and our PRS model was proven to be proinflammatory, evidenced by increased systemic IL-6 (Vanbesien-Mailliot et al., 2007). Various studies have shown that sialic acids play a major role in inflammation by upregulating anti-inflammatory cytokines IL-4 and IL-10 (Payazdan et al., 2021). The loss of circulatory glycoprotein α2,6 sialylation resulted in significant changes in metabolic pathways, boosting inflammatory cytokine production (Oswald et al., 2020). Transcriptional changes don't always reflect enzyme activity, so sialyltransferase gene expression isn't a reliable indicator of polysialylation, for this reason studies using lectin blotting are needed to assess polysialylation activity.

Overall, this study showed that gestational stress programmed PRS offspring at behavioral and hormonal levels, resulting in reduced risk-taking behavior and increased plasma CORT levels in response to novelty stress, showing impaired HPA axis response. Treatment in their F0 mothers rescued these phenotypes, underscoring the role of probiotic strains like *L. reuteri* in controlling behavior and hormonal balance, and highlighting the gut-brain axis efficiency. OTR, proBDNF, BDNF, and sialyltransferase expression were impacted by stress, but *L. reuteri* could not fully rescue this imbalance. Further investigations will include systemic cytokine measurements through a collaboration with Pr. Boualem Sendid's lab (UGSF) to investigate inflammation directly in the gut of these offspring. Additionally, evaluating bacterial populations in the gut through 16S sequencing is necessary to know if this strain has an impact on the gut microbiota populations.

**REFERENCES**

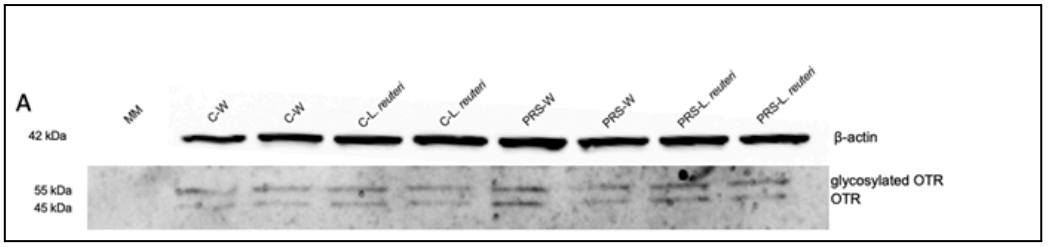

1) Abe, C., Yi, Y., Hane, M., Kitajima, K., Sato, C., 2019. Acute stress-induced change in polysialic acid levels mediated by sialidase in mouse brain. Sci. Rep. 9, 9950. https://doi.org/10.1038/s41598-019-46240-6
2) Amini-Khoei, H., Mohammadi-Asl, A., Amiri, S., Hosseini, M.-J., Momeny, M., Hassanipour, M., Rastegar, M., Haj-Mirzaian, A., Mirzaian, A.H.-, Sanjarimoghaddam, H., Mehr, S.E., Dehpour, A.R., 2017. Oxytocin mitigated the depressive-like behaviors of maternal separation stress through modulating mitochondrial function and neuroinflammation. Prog. Neuropsychopharmacol. Biol. Psychiatry 76, 169–178. https://doi.org/10.1016/j.pnpbp.2017.02.022
3) Bermúdez-Humarán, L.G., Salinas, E., Ortiz, G.G., Ramirez-Jirano, L.J., Morales, J.A., Bitzer-Quintero, O.K., 2019. From Probiotics to Psychobiotics: Live Beneficial Bacteria Which Act on the Brain-Gut Axis. Nutrients 11, 890. https://doi.org/10.3390/nu11040890
4) Carter, C.S., Kenkel, W.M., MacLean, E.L., Wilson, S.R., Perkeybile, A.M., Yee, J.R., Ferris, C.F., Nazarloo, H.P., Porges, S.W., Davis, J.M., Connelly, J.J., Kingsbury, M.A., 2020. Is Oxytocin "Nature's Medicine"? Pharmacol. Rev. 72, 829–861. https://doi.org/10.1124/pr.120.019398
5) Cryan, J.F., Dinan, T.G., 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 13, 701–712. https://doi.org/10.1038/nrn3346
6) Dinan, T.G., Stanton, C., Cryan, J.F., 2013. Psychobiotics: A Novel Class of Psychotropic. Biol. Psychiatry, Novel Therapeutics for Depression 74, 720–726. https://doi.org/10.1016/j.biopsych.2013.05.001
7) Dirven, B.C.J., Homberg, J.R., Kozicz, T., Henckens, M.J. a. G., 2017. Epigenetic programming of the neuroendocrine stress response by adult life stress. J. Mol. Endocrinol. 59, R11–R31. https://doi.org/10.1530/JME-17-0019
8) Friuli, M., Eramo, B., Valenza, M., Scuderi, C., Provensi, G., Romano, A., 2021. Targeting the Oxytocinergic System: A Possible Pharmacological Strategy for the Treatment of Inflammation Occurring in Different Chronic Diseases. Int. J. Mol. Sci. 22, 10250. https://doi.org/10.3390/ijms221910250
9) Gatta, E., Mairesse, J., Deruyter, L., Marrocco, J., Van Camp, G., Bouwalerh, H., Lo Guidice, J.-M., Morley-Fletcher, S., Nicoletti, F., Maccari, S., 2018. Reduced maternal behavior caused by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance. NeuroToxicology 66, 138–149. https://doi.org/10.1016/j.neuro.2018.04.005
10) Hoarau, G., Mukherjee, P.K., Gower-Rousseau, C., Hager, C., Chandra, J., Retuerto, M.A., Neut, C., Vermeire, S., Clemente, J., Colombel, J.F., Fujioka, H., Poulain, D., Sendid, B.,

Ghannoum, M.A., 2016. Bacteriome and Mycobiome Interactions Underscore Microbial Dysbiosis in Familial Crohn's Disease. mBio 7, 10.1128/mbio.01250-16. https://doi.org/10.1128/mbio.01250-16

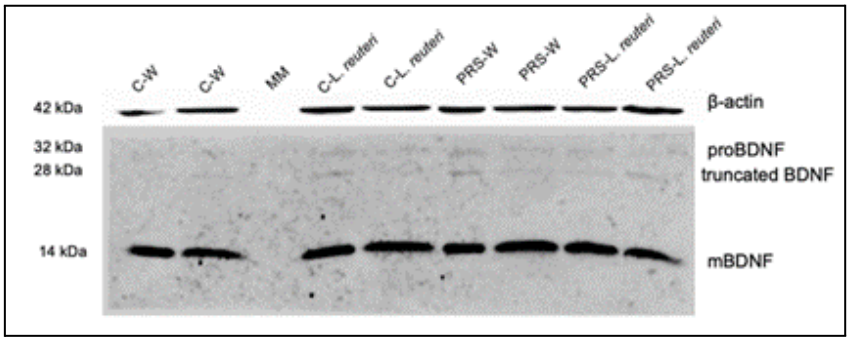
11) Kimura, T., Makino, Y., Bathgate, R., Ivell, R., Nobunaga, T., Kubota, Y., Kumazawa, I., Saji, F., Murata, Y., Nishihara, T., Hashimoto, M., Kinoshita, M., 1997. The role of N-terminal glycosylation in the human oxytocin receptor. Mol. Hum. Reprod. 3, 957–963. https://doi.org/10.1093/molehr/3.11.957
12) Krick, S., Helton, E.S., Easter, M., Bollenbecker, S., Denson, R., Zaharias, R., Cochran, P., Vang, S., Harris, E., Wells, J.M., Barnes, J.W., 2021. ST6GAL1 and α2-6 Sialylation Regulates IL-6 Expression and Secretion in Chronic Obstructive Pulmonary Disease. Front. Immunol. 12. https://doi.org/10.3389/fimmu.2021.693149
13) Maccari, S., Polese, D., Reynaert, M.-L., Amici, T., Morley-Fletcher, S., Fagioli, F., 2017. Early-life experiences and the development of adult diseases with a focus on mental illness: The Human Birth Theory. Neuroscience 342, 232–251. https://doi.org/10.1016/j.neuroscience.2016.05.042
14) Mairesse, J., Gatta, E., Reynaert, M.-L., Marrocco, J., Morley-Fletcher, S., Soichot, M., Deruyter, L., Camp, G.V., Bouwalerh, H., Fagioli, F., Pittaluga, A., Allorge, D., Nicoletti, F., Maccari, S., 2015. Activation of presynaptic oxytocin receptors enhances glutamate release in the ventral hippocampus of prenatally restraint stressed rats. Psychoneuroendocrinology 62, 36–46. https://doi.org/10.1016/j.psyneuen.2015.07.005
15) Morley-Fletcher, S., Gaetano, A., Gao, V., Gatta, E., Van Camp, G., Bouwalerh, H., Thomas, P., Nicoletti, F., Maccari, S., 2024. Postpartum Oxytocin Treatment via the Mother Reprograms Long-Term Behavioral Disorders Induced by Early Life Stress on the Plasma and Brain Metabolome in the Rat. Int. J. Mol. Sci. 25, 3014. https://doi.org/10.3390/ijms25053014
16) Morley-Fletcher, S., Mairesse, J., Soumier, A., Banasr, M., Fagioli, F., Gabriel, C., Mocaer, E., Daszuta, A., McEwen, B., Nicoletti, F., Maccari, S., 2011. Chronic agomelatine treatment corrects behavioral, cellular, and biochemical abnormalities induced by prenatal stress in rats. Psychopharmacology (Berl.) 217, 301–313. https://doi.org/10.1007/s00213-011-2280-x
17) Oswald, D.M., Jones, M.B., Cobb, B.A., 2020. Modulation of hepatocyte sialylation drives spontaneous fatty liver disease and inflammation. Glycobiology 30, 346–359. https://doi.org/10.1093/glycob/cwz096
18) Patterson, E., Ryan, P.M., Wiley, N., Carafa, I., Sherwin, E., Moloney, G., Franciosi, E., Mandal, R., Wishart, D.S., Tuohy, K., Ross, R.P., Cryan, J.F., Dinan, T.G., Stanton, C., 2019. Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome. Sci. Rep. 9, 16323. https://doi.org/10.1038/s41598-019-51781-x
19) Payazdan, M., Khatami, S., Galehdari, H., Delfan, N., Shafiei, M., Heydaran, S., 2021. The

anti-inflammatory effects of sialic acid on the human glia cells by the upregulation of *IL-4* and *IL-10* genes' expressions. Gene Rep. 24, 101218. https://doi.org/10.1016/j.genrep.2021.101218

20) Pesarico, A.P., Bueno-Fernandez, C., Guirado, R., Gómez-Climent, M.Á., Curto, Y., Carceller, H., Nacher, J., 2019. Chronic Stress Modulates Interneuronal Plasticity: Effects on PSA-NCAM and Perineuronal Nets in Cortical and Extracortical Regions. Front. Cell. Neurosci. 13, 197. https://doi.org/10.3389/fncel.2019.00197
21) Poutahidis, T., Kearney, S.M., Levkovich, T., Qi, P., Varian, B.J., Lakritz, J.R., Ibrahim, Y.M., Chatzigiagkos, A., Alm, E.J., Erdman, S.E., 2013. Microbial Symbionts Accelerate Wound Healing via the Neuropeptide Hormone Oxytocin. PLoS ONE 8, e78898. https://doi.org/10.1371/journal.pone.0078898
22) Sandi, C., Merino, J.J., Cordero, M.I., Touyarot, K., Venero, C., 2001. Effects of chronic stress on contextual fear conditioning and the hippocampal expression of the neural cell adhesion molecule, its polysialylation, and L1. Neuroscience 102, 329–339. https://doi.org/10.1016/s0306-4522(00)00484-x
23) Tret'yakova, L.V., Kvichansky, A.A., Barkovskaya, E.S., Manolova, A.O., Bolshakov, A.P., Gulyaeva, N.V., 2023. Ambiguous Contribution of Glucocorticosteroids to Acute Neuroinflammation in the Hippocampus of Rat. Int. J. Mol. Sci. 24, 11147. https://doi.org/10.3390/ijms241311147
24) Vanbesien-Mailliot, C.C.A., Wolowczuk, I., Mairesse, J., Viltart, O., Delacre, M., Khalife, J., Chartier-Harlin, M.-C., Maccari, S., 2007. Prenatal stress has pro-inflammatory consequences on the immune system in adult rats. Psychoneuroendocrinology 32, 114–124. https://doi.org/10.1016/j.psyneuen.2006.11.005
25) Varian, B.J., Poutahidis, T., DiBenedictis, B.T., Levkovich, T., Ibrahim, Y., Didyk, E., Shikhman, L., Cheung, H.K., Hardas, A., Ricciardi, C.E., Kolandaivelu, K., Veenema, A.H., Alm, E.J., Erdman, S.E., 2017. Microbial lysate upregulates host oxytocin. Brain. Behav. Immun. 61, 36–49. https://doi.org/10.1016/j.bbi.2016.11.002
26) Verhaeghe, R., Gao, V., Morley-Fletcher, S., Bouwalerh, H., Van Camp, G., Cisani, F., Nicoletti, F., Maccari, S., 2021. Maternal stress programs a demasculinization of glutamatergic transmission in stress-related brain regions of aged rats. GeroScience 44, 1047–1069. https://doi.org/10.1007/s11357-021-00375-5
27) Yang, J., Harte-Hargrove, L.C., Siao, C.-J., Marinic, T., Clarke, R., Ma, Q., Jing, D., Lafrancois, J.J., Bath, K.G., Mark, W., Ballon, D., Lee, F.S., Scharfman, H.E., Hempstead, B.L., 2014. proBDNF negatively regulates neuronal remodeling, synaptic transmission, and synaptic plasticity in hippocampus. Cell Rep. 7, 796–806. https://doi.org/10.1016/j.celrep.2014.03.040




**Supplementary Figure 1. Representative immunoblot of OTR (45 kDa) and glycosylated OTR (55 kDa) in the dorsal hippocampus of the male progeny.** (A) Immunoblots targeting OTR and glycosylated OTR. C-W = Control-Water, C-L. *reuteri* = Control-L. *reuteri*, PRS-W = PRS-Water, PRS-L.*reuteri*, MM = molecular marker.




**Supplementary Figure 2. Representative immunoblot of mBDNF (14 kDa) and proBDNF (32 kDa) in the ventral hippocampus of the male PRS progeny.** Immunoblots were performed in duplicate. C-W = Control-Water, C-L. *reuteri* = Control-L. *reuteri*, PRS-W = PRS-Water, PRS-L.*reuteri*, MM = molecular marker.

# General discussion

# General discussion

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This PhD thesis investigated how gestational stress could affect future generations through epigenetic mechanisms. I focused on how a mother's behavior could influence this process and explored potential solutions like carbetocin (CBT) and the probiotic *Limosilactobacillus reuteri (L. reuteri)* to mitigate stress effects. By concentrating on the *postpartum* period, my study aimed to develop specific strategies that could improve long-term health outcomes and break the cycle of stress vulnerability across generations. My thesis addressed gaps in behavioral epigenetics by emphasizing the critical role of oxytocin (OT) in early-life programming. While the precise mechanisms of oxytocin's beneficial effects remained incompletely understood, our findings indicated that oxytocinergic activation acted through its anti-stress effects and enhancement of maternal behavior. This suggested promising therapeutic approaches targeting the *postpartum* period using CBT or *L. reuteri*, offering potential for reversing stress programming and highlighting the epigenetic aspects of early-life stress.

Specifically, in the current study using the perinatal stress model (PRS), we highlighted that gestational stress occurring in F0 dams programs the offspring, across multiple generations in an intergenerational and transgenerational manner, more specifically at the level of the stress/anti-stress balance and HPA axis. We put a specific accent on maternal behavior hypothesizing that it mediated the transmitted effects. Remarkably, increasing maternal behavior in F0 mothers through *postpartum* CBT broke the transmission of PRS deficits up to F3 male offspring. In the hippocampus, prominent biomarkers were impacted by the PRS, specifically the ones implicated in stress/anti-stress balance and HPA axis activity, such as CORT, OT, MR, GR, BDNF and mGluRs.

Moreover, this investigation filled the gaps and the few knowledge we had in the process happening in the mothers, we showed that stress/anti-stress balance in dams and a multitude of behavioral deficits were critically impaired in stressed and PRS dams over the generations, some of these deficits started even before parturition such as impaired nest building in stress dams, extending to imbalance in the equilibrium of plasmatic CORT and OT levels persisting few days after the weaning.

Additionally to the pharmacological approach using CBT, we generated robust and promising results by activating oxytocinergic pathways, though a non-pharmacological treatment using the probiotic *L. reuteri* in the first *postpartum* week. We proved that this strain could rescue maternal care and stress/anti-stress balance in the plasma and in the hypothalamus; an important brain region for OT synthesis and maternal behavior. We highlighted the implication of BDNF/OTR balance in the beneficial effects of the probiotic.

Our findings strikingly revealed deficits in maternal behaviors during the *postpartum* period, which were not significant after weaning. Indeed, post-weaning risk-taking behavior was unaffected, which bears similarities to *postpartum* depression (PPD) in humans, characterized by sadness, anxiety, mood instability, and episodes of crying due to the rapid hormonal and psychological adjustments occurring in the first weeks after childbirth. Furthermore, dams treated with the probiotic *L. reuteri* produced offspring with corrected deficits in CORT levels and risk-taking behavior in adult PRS males . However, our preliminary results indicated that in the ventral hippocampus, the probiotic treatment did not affect the offspring's BDNF, OTR, and sialylation parameters. Further investigations are needed in both males and females.

*Overall, our findings highlight the pivotal role of maternal behavior and the oxytocinergic system, which can interrupt the transmission of stress and transform it into a chain of beneficial effects.*

### *I. Epigenetic inheritance of the PRS*

In the first part of this thesis, we hypothesized that gestational stress could be stably transmitted through intergenerational and transgenerational pathways mediated by maternal behavior. Secondly, we postulated that the transmission can be broken through enhancing maternal behavior with oxytocinergic activation (using CBT IP or IN). Our findings are widely corroborated in the literature. Referring back to Seymour Levine's pioneering 1957 work on early life stress, specifically the "early handling" model in neonatal rats, which demonstrated that brief handling could induce lasting reductions in stress reactivity and alter neuroendocrine function, particularly within the HPA axis. This research has been instrumental in understanding how early environmental factors shape stress physiology and behavior across the lifespan (Levine, 1957). Later on, several studies in rodents and humans have proven that stress during critical windows such as the prenatal (gestation) or postnatal

(*postpartum*) periods induces intergenerational and transgenerational transmission of stress-related deficits. This has been shown in rodents (Gapp et al., 2014; Perez and Lehner, 2019; Sandovici et al., 2022) and humans (Bowers and Yehuda, 2016; Tolkunova et al., 2023). Other studies have highlighted the accumulation of transmitted deficits, showing worsening over generations, such as reduced maternal care, impaired inflammation, and blood-brain barrier dysfunction up to the F2 generation (Nephew et al., 2017). Similarly, in human PTSD, symptoms worsen with age and time (Yehuda et al., 2009). The mechanisms of early-life stress programming have been extensively investigated. One of the earliest studies demonstrated that maternal-mediated programming in offspring was reflected in increased corticosterone (CORT) levels in the pups of stressed dams (Angelucci et al., 1985). Later, stressed mothers were classified as high or low corticosterone mothers, with high CORT mothers producing offspring with persistent CORT levels after restraint stress and reduced hippocampal MR expression (Barbazanges et al., 1996). In the PRS model, it was shown that deficits in PRS offspring were reversed when adoption occurred within the first 3-6 hours after birth (Maccari et al., 1995). Like that Maccari's work provided early evidence of maternal-mediated mechanisms and highlighted maternal care as a pivotal component, paving the way for behavioral epigenetics. Nearly 10 years later, pioneering research by Prof. Moshe Szyf and Prof. Michael Meaney directly linked maternal behavior to epigenetic regulation of GR through its increased methylation in offspring from low licking-grooming/arched-back nursing (LG/ABN) dams (Weaver et al., 2004). Additionally, Weaver and collaborators used Trichostatin A (TSA), a histone deacetylase inhibitor that reduces the accessibility of DNA methyltransferases (DNMTs) to DNA, potentially reducing DNA methylation. The results showed that low LG/ABN treated with TSA mitigated HPA axis deficits in offspring (Weaver et al., 2004). Conversely, increasing DNA methylation of the GR promoter in high LG/ABN through L-Methionine led to HPA axis alterations in offspring (Weaver et al., 2005). This led to multiple investigations into the role of epigenetics in stress programming and its transmission, exploring mechanisms such as histone modifications and microRNAs (Jarmasz et al., 2019). These mechanisms are complex and interrelated, as illustrated by Conrad Waddington's concept of the "epigenetic landscape," which metaphorically describes how gene expression is influenced by interactions between genes and the environment during development (Waddington, 1942).

Our investigation confirmed the role of maternal behavior through oxytocinergic intervention. We demonstrated that early interventions during the first week of the *postpartum* period increased maternal care, and that was sufficient to rescue early-life stress deficits and break intergenerational and transgenerational transmission. This study is the first of its kind to show the reversal of stress-related perturbations using oxytocinergic activation and enhanced maternal behavior through multiple generations. This approach was based on previous reports of the PRS model, which demonstrated significant potential for mitigating stress-related deficits through *postpartum* CBT administration in mothers. These studies indicated that *postpartum* CBT could correct behavioral, neurochemical, and metabolic deficits in adult PRS offspring, with these improvements persisting into old age (Gatta et al., 2018; Morley-Fletcher et al., 2024).

However, maternal behavior and related factors to maternal environment are not the only programming factors, the influence of the germline, cannot be denied. One study has presented an impact of maternal preconception stress on oocytes (Zaidan et al., 2013). On the other hand, Mansuy's lab showed through their postnatal maternal separation and unpredictable stress (MSUS) model that the paternal line transmitted behavioral and molecular deficits across generations, accompanied by permanent deficits in the germline (sperm) (Gapp et al., 2014; Rodgers et al., 2015). These findings show similarities with previous data in the maternal line (Roth et al., 2009; Weaver et al., 2005, 2004) and illustrate the broad detrimental impact of early stress.

In the context of this PhD, our work has revealed that stress transmission can be disrupted through early interventions with CBT, creating a beneficial chain of transmission across generations. Specifically, we showed intraperitoneal intergenerational correction with CBT IP and transgenerational transmission with both CBT IP and CBT IN activations. In the present study we did not aim to compare between both route, since they use separate methodologies and dosages that are based on two different reports, for CBT IP we used the methodology published from Gatta and Morley-fletcher articles (Gatta et al., 2018; Morley-Fletcher et al., 2024), for the internal part we adapted our protocol according to Calcagnoli's work on OT (Calcagnoli et al., 2015). Notably, the intranasal route appears to be more practical and effective in clinical applications, since OT through this route has already been reported to directly access the brain through the olfactory nerves and tegmental nerves (Yao and

Kendrick, 2022), and can be absorbed and detected in the brain (Lee et al., 2020). For example, insecure individuals show improved emotional, behavioral, and neural responses to infant crying after intranasal OT treatment (Riem et al., 2016). Furthermore, this route of administration has been shown to reduce amygdala activity when listening to an infant's crying (Riem et al., 2011), and enhances stress-protective effects of social support in women with negative childhood experiences (Riem et al., 2020). Additionally, in preclinical rat models it attenuates stress responses following chronic unpredictible stress in rats (Yang et al., 2019). Furthermore, CBT represents a more stable analog of oxytocin with a longer half-life (Jin et al., 2019), which does not require cold-chain transport and storage, reducing logistical challenges and costs (Jin et al., 2019; Gil-Rojas et al., 2018). These attributes make oxytocinergic therapy a promising candidate for treating stress-related disorders.

### *II. The anti-stress effect of Limosilactobacillus reuteri in mothers-offspring dyad*

Based on the initial hypothesis which put hope on the oxytocinergic activation in reversing stress transmission in mothers-offspring dyad, we tried an alternative approach through probiotic supplementations of *Limosilactobacillus reuteri (L. reuteri)*, during the first *postpartum* week. In this part of the thesis we hypothesized that *L. reuteri* could mitigate stress-deficits though oxytocinergic activation. Primary, we characterized extensively the treated mothers and the responsiveness of *L. reuteri* to correct the stressed phenotype. We showed that the reduced maternal care by gestational stress was increased by *L. reuteri* treatment, and the treatment enhanced systemic and hypothalamic OT levels. These findings were supported by robust reports in the literature, where the strain increased OT, reduced CORT, enhanced social behavior, and normalized inflammation response. As advanced in the beginning of this discussion, the abnormalities observed in the *postpartum* period recapitulated deficits similar to *postpartum* depression (PPD), and the anti-stress effect of *L. reuteri* can be inserted in the criteria of the WHO that preconize to work on the health of both the child and the mother and the need to develop alternative strategies. Moreover, what is interesting with this probiotic approach is the fact that it's less invasive, natural, and less risk of secondary effects. Indeed, *L. reuteri* is naturally present in the gut but also in fermented food, in fact *L. reuteri* can successfully grow and remain stable in non-dairy fermented beverages. The bacteria can reach high viable counts, even after extended storage periods, indicating that such fermented products can serve as good carriers for probiotics like *L.*

*reuteri* (Mauro et al., 2016). For instance, a study published in *Nutrients* discussed how various strains of Lactobacillus, including *L. reuteri*, can influence the levels of neurotransmitters in the brain and improve mood-related behaviors in animal models (Xiong et al., 2023). Additionally, research published in *Frontiers in Psychology* details how *L. reuteri* contributes to maintaining homeostasis of the immune system and promoting stress resilience through the regulation of IFNγ levels (Merchak et al., 2024). Moreover, fermented foods such as yogurt, kefir, and certain cheeses are known to contain beneficial strains of *Lactobacillus*, including *L. reuteri*. These foods are traditionally consumed in various cultures and have been linked to improved gut health and mental well-being. A review in the *Journal of Physiological Anthropology* outlines how fermented foods, by enhancing the gut microbiota, can reduce inflammation and support mental health (Selhub et al., 2014; Wastyk et al., 2021). Indeed, researchers showed that a Lactobacillus strain derived from fermented cabbage (kimchi) can improve mental functioning and hippocampal BDNF production in animals (Jung et al., 2012).

Surprisingly, offspring of probiotic-treated mothers showed corrected risk-taking behavior and CORT imbalance, although neurochemical parameters (sialyltransferases) in the ventral hippocampus were less responsive to the probiotic treatment. The exact mechanisms by which probiotics exert their effects remain complex, among the advanced mechanisms in the introduction, anti-inflammation effect, neurotransmitters enhancement, and metabolites production, all within the context of the gut-brain axis, a novel theory suggests the possibility of a brain microbiota. This concept is intriguing the scientific community as it challenges the longstanding belief that the brain is a sterile organ, protected from harmful agents circulating in the blood. Neuroscientists and microbiologists rarely work together, which has contributed to the oversight of the potential brain microbiota. Additionally, confirming the presence of microbes in the brain is difficult because techniques often rely on analyzing foreign genetic material, which can be unreliable due to contamination. The initial suggestion of native bacteria in the brain came from a 2013 study on microbial infiltration in the brains of HIV/AIDS patients. Branton et al. observed non-human RNA sequences aligning with over 170 bacteria and phages, suggesting a previously unrecognized brain microbiota (Branton et al., 2013).

Indeed, while epigenetic modifications often monopolize the focus when investigating early-life stress (ELS) programming, other post-translational mechanisms, such as glycosylation processes, also play a significant role. For example, prenatally stressed fetuses often have lower plasma glucose levels, suggesting reduced transplacental glucose transfer, a study demonstrated that maternal chronic stress suppresses the expression of placental O-GlcNAc transferase (OGT), a nutrient-sensitive protein that post-translationally modified proteins associated with histone remodeling, which has broad functions in brain development. The offspring of a mouse model lacking placental OGT completely recapitulated the prenatal stress phenotype, underscoring OGT's and post-translational modifications role in modulating prenatal stress effects (Howerton and Bale, 2014). In the brain of PRS offspring, the expression of glycosylation (sialylation) enzymes ST8SIA4 and ST6GAL1 appeared perturbed, with these changes remaining unmitigated by *L. reuteri* treatment. As discussed in the last chapter of the results, our exploratory aim was to uncover connections between seemingly disparate parameters that exert individual effects. This novel approach extends beyond classical epigenetic programming in ELS by integrating post-translational modifications, such as sialylation.

***Taken together,*** both *postpartum* CBT and *L. reuteri* enhance maternal care, however as explained in the introduction, behaviors are complex phenotypes that combine both hormonal and molecular components, and stressful events occurring during gestation encounter two primary barriers that protect the fetus from mal-programming. The first barrier is the negative feedback mechanism of the HPA axis, which aims to downregulate glucocorticoid production. The second line of defense is the placenta, which expresses the enzyme 11β-HSD2. This enzyme degrades glucocorticoids passing through the placenta, providing a crucial checkpoint (Brunton and Russell, 2008; Sze and Brunton, 2024). The effectiveness of these barriers is questioned in the context of early-life stress (ELS), which is the main subject of this thesis. As highlighted in the introduction, gestational stress alters the HPA axis as well as the expression of 11β-HSD2. However, even if the previously mentioned protective mechanisms are considered effective, a new route of glucocorticoid transmission emerges during the lactation period following gestation. Previous research in the PRS model has already shown that maternal milk mediates the glucocorticoid impact on the pups (Barbazanges et al., 1996). Moreover, Angelucci's work highlighted the critical significance

of maternal hormones as key mediators in shaping health and behavioral trajectories across the lifespan (Angelucci et al., 1985). Additionally, a clinical study on maternal stress showed a high correlation between salivary cortisol and human milk cortisol during the first week of lactation (Rosen-Carole et al., 2024), additionally, perinatal psychosocial stress negatively affected milk composition such as fat and saturated fatty acids in milk (Ziomkiewicz et al., 2021). While we did not evaluate milk composition in the present study, we assessed maternal care by observing active maternal behaviors such as nursing and arched-back nursing. This approach ensured a qualitative analysis of behavioral indicators of lactation.

Overall, the stability of stress over one or multiple generations is a subject of debate, some diverging points of view consider that this transmission is contradictory with the concept of epigenetic itself and its reversibility, and some scientists think that the persistence of such phenotypes and deficits is due to very controlled and restricted experimental conditions that favor the chain of transmission, and in human the cultural and social component can favor the persistence of stress blueprints. However the investigation of such transmission in humans was initiated in the Holocaust survivors, the concept of intergenerational trauma was introduced in the psychiatric literature through descriptions of behavioral and clinical problems in offspring of Holocaust survivors (Rakoff, 1966; Weinfeld et al., 1981).

## Conclusion

The work of my PhD highlighted the wide impact of early-life stress and the crucial role of maternal behavior in offspring programming through both intergenerational and transgenerational mechanisms. This was demonstrated through *postpartum* treatments (CBT or Probiotic), which underscored the potential to reverse stress-induced deficits when interventions occurred during early stages of development. Key findings from this research include: **1)** Intergenerational inheritance of early-life stress is mediated by maternal oxytocin. **2)** PRS-related deficits are transmitted transgenerationally to F3 males, but *postpartum* carbetocin can break this inheritance. **3)** *Postpartum L. reuteri* corrects maternal behavior through a mechanism involving BDNF and OT balance in the hypothalamus. **4)** PRS programming in male offspring is partially mitigated by *postpartum L. reuteri* treatment in the mothers, but still further investigations in the offspring are needed.

## Perspectives

While this study provides valuable insights, it also represents the beginning of new perspectives and avenues for investigations. Further research is needed to explore if our *postpartum* treatments could have preventive effects, and whether animals treated beforehand remain resilient to stress-related deficits when exposed later. Furthermore, one unresolved question remains: does CBT treatment directly target the same epigenetic mechanisms as PRS, such as GR, MR, and BDNF promoter methylations? This question can be addressed through DNA methylation analysis of offspring from dams treated with CBT, particularly focusing on the F3 generation, which was not directly exposed to stress. MeDIP (Methylated DNA Immunoprecipitation) analysis is a suitable technique for this purpose, allowing us to examine changes in DNA methylation patterns, providing insights into potential epigenetic modifications induced by the treatments, and deepen our understanding of the molecular mechanisms underlying the therapeutic effects of oxytocinergic activation.

Additionally, intriguing sex differences in risk-taking behavior were noted, despite similar neurochemical profiles showing increased CORT and reduced OT in both PRS males and females. This suggests the involvement of sex hormones, possibly estrogen, because estrogen is tightly linked to OT through estrogen response elements (EREs), localized in the OT gene promoter region. Finally, while epigenetic modifications are the classical mechanisms investigated, they do not represent the sole route of transmission. The role of the germline in this transmission chain cannot be overlooked. To explore this possibility, in the maternal line we can investigate potential impacted targets in the ovaries. Since the specific targets are not well identified in the germline of our PRS model, RNA sequencing techniques offer an ideal approach to screen a multitude of pathways.

The *postpartum* probiotic *L. reuteri* has shown promising results, yet the mechanisms underlying its effects on the brain and its enhancement of oxytocinergic activation remain unresolved. There are two possibilities to consider. First, *L. reuteri* may secrete molecules, for example *reuterin*, that act on both the brain and periphery to enhance OT levels. Second, there is a theoretical possibility that the bacteria itself can reach the brain to exert its effects, although this has never been demonstrated. Most neuroscientists are skeptical about the existence of a *brain microbiota*, as it has never been confirmed clearly, in fact it is

challenging to demonstrate it due to potential contamination during dissection. To investigate the presence of a brain microbiota, one suitable technique is 16S rRNA gene sequencing, which consists of sequencing the specific bacterial 16S rRNA gene, thus providing information about the microbial composition present.

*Finally, as highlighted at the beginning of this manuscript, our study aligns with the Developmental Origins of Health and Disease (DOHaD) theory, focusing on critical windows of development such as the gestation and postpartum periods, by emphasizing the promising role of postpartum oxytocinergic activation to mitigate stress-related deficits. According to recommendations from the World Health Organization (WHO), these critical developmental windows extend to include the first 1000 days of life. However, current clinical practices often rely on anxiolytics for managing anxiety and stress, which can pose challenges in early life due to side effects and sensitivity of the developing brain. Effective treatment for anxiety and stress disorders requires a multifaceted approach including social buffering for example cognitive behavioral therapy, early interventions like telephone-delivered therapy, programs such as the Nurse-Family Partnership which reduce maternal stress and depression, and social support interventions. Among these strategies our findings can be inserted easily, at least for the probiotic approach. Regarding CBT further investigations could be done, but the fact that CBT is already used for medical purposes of postpartum hemorrhage makes it more acceptable.*

*The insights advanced in this study hold potential promises to enhance mental health outcomes in populations affected by historical traumas such as colonization, slavery, and displacement, as observed in North African countries where elevated rates of PTSD are prevalent, sometimes referred to as the North African Syndrome. Applying these findings could lead to more effective mental health strategies tailored to address early-life stress and its transgenerational transmission. Such approaches can also be extended to other vulnerable populations worldwide facing similar challenges, fostering resilience and promoting mental health recovery on a broader scale.*

## References


1) Ainsworth, M.D.S., Blehar, M.C., Wall, S., Waters, E., 1978. Patterns of Attachment: A Psychological Study of the Strange Situation. Lawrence Erlbaum Associates.

2) Alves, R.L., Portugal, C.C., Lopes, I.M., Oliveira, P., Alves, C.J., Barbosa, F., Summavielle, T., Magalhães, A., 2022. Maternal stress and vulnerability to depression: coping and maternal care strategies and its consequences on adolescent offspring. Transl. Psychiatry 12, 463. https://doi.org/10.1038/s41398-022-02220-5
3) Angelucci, L., Patacchioli, F.R., Scaccianoce, S., Di Sciullo, A., Cardillo, A., Maccari, S., 1985. A model for later-life effects of perinatal drug exposure: maternal hormone mediation. Neurobehav. Toxicol. Teratol. 7, 511–517.
4) Avendano, S., Moirangthem, S., Taflet, M., Heude, B., Koehl, M., van der Waerden, J., Downes, N., 2024. Prenatal maternal negative life events associated with child emotional and behavioral problems in the French EDEN cohort. J. Affect. Disord. 356, 224–232. https://doi.org/10.1016/j.jad.2024.04.040
5) Bale, T.L., Baram, T.Z., Brown, A.S., Goldstein, J.M., Insel, T.R., McCarthy, M.M., Nemeroff, C.B., Reyes, T.M., Simerly, R.B., Susser, E.S., Nestler, E.J., 2010. Early Life Programming and Neurodevelopmental Disorders. Biol. Psychiatry 68, 314–319. https://doi.org/10.1016/j.biopsych.2010.05.028
6) Barbazanges, A., Piazza, P.V., Moal, M.L., Maccari, S., 1996. Maternal Glucocorticoid Secretion Mediates Long-Term Effects of Prenatal Stress. J. Neurosci. 16, 3943–3949. https://doi.org/10.1523/JNEUROSCI.16-12-03943.1996
7) Barker, 1995a. Fetal origins of coronary heart disease. BMJ 311, 171–174.
8) Barker, 1995b. Intrauterine programming of adult disease. Mol. Med. Today 1, 418–423. https://doi.org/10.1016/S1357-4310(95)90793-9
9) Barker, D.J., 1990. The fetal and infant origins of adult disease. BMJ 301, 1111.
10) Barker, D.J.P., 2004. The developmental origins of well-being. Philos. Trans. R. Soc. B Biol. Sci. 359, 1359–1366. https://doi.org/10.1098/rstb.2004.1518
11) Barker, D.J.P., Osmond, C., 1986. barker et al 1986 epidimiology study.
12) Bell, A.F., Carter, C.S., Steer, C.D., Golding, J., Davis, J.M., Steffen, A.D., Rubin, L.H., Lillard, T.S., Gregory, S.P., Harris, J.C., Connelly, J.J., 2015. Interaction between oxytocin receptor DNA methylation and genotype is associated with risk of postpartum depression in women without depression in pregnancy. Front. Genet. 6, 243. https://doi.org/10.3389/fgene.2015.00243

13) Berger, S.L., Kouzarides, T., Shiekhattar, R., Shilatifard, A., 2009. An operational definition of epigenetics. Genes Dev. 23, 781–783. https://doi.org/10.1101/gad.1787609

14) Berkowitz, G.S., Wolff, M.S., Janevic, T.M., Holzman, I.R., Yehuda, R., Landrigan, P.J., 2003. The World Trade Center disaster and intrauterine growth restriction. JAMA 290, 595–596. https://doi.org/10.1001/jama.290.5.595-b

15) Bertram, C., Khan, O., Ohri, S., Phillips, D.I., Matthews, S.G., Hanson, M.A., 2008. Transgenerational effects of prenatal nutrient restriction on cardiovascular and hypothalamic-pituitary-adrenal function. J. Physiol. 586, 2217–2229. https://doi.org/10.1113/jphysiol.2007.147967

16) Boscardin, C., Manuella, F., Mansuy, I.M., 2022. Paternal transmission of behavioural and metabolic traits induced by postnatal stress to the 5th generation in mice. Environ. Epigenetics 8, dvac024. https://doi.org/10.1093/eep/dvac024

17) Bosch, O.J., 2013. Maternal aggression in rodents: brain oxytocin and vasopressin mediate pup defence. Philos. Trans. R. Soc. B Biol. Sci. 368, 20130085. https://doi.org/10.1098/rstb.2013.0085

18) Bowers, M.E., Yehuda, R., 2016. Intergenerational Transmission of Stress in Humans. Neuropsychopharmacology 41, 232–244. https://doi.org/10.1038/npp.2015.247

19) Bowlby, J., 1969. Attachment and Loss. Basic Books.

20) Branchi, I., Curley, J.P., D'Andrea, I., Cirulli, F., Champagne, F.A., Alleva, E., 2013. Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. Psychoneuroendocrinology 38, 522–532. https://doi.org/10.1016/j.psyneuen.2012.07.010

21) Branton, W.G., Ellestad, K.K., Maingat, F., Wheatley, B.M., Rud, E., Warren, R.L., Holt, R.A., Surette, M.G., Power, C., 2013. Brain Microbial Populations in HIV/AIDS: α-Proteobacteria Predominate Independent of Host Immune Status. PLOS ONE 8, e54673. https://doi.org/10.1371/journal.pone.0054673

22) Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., Cryan, J.F., 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc. Natl. Acad. Sci. 108, 16050–16055. https://doi.org/10.1073/pnas.1102999108

23) Bray, N., 2019. The microbiota–gut–brain axis. Nat. Res. https://doi.org/10.1038/d42859-019-00021-3

24) Breton, C.V., Landon, R., Kahn, L.G., Enlow, M.B., Peterson, A.K., Bastain, T., Braun, J., Comstock, S.S., Duarte, C.S., Hipwell, A., Ji, H., LaSalle, J.M., Miller, R.L., Musci, R., Posner, J., Schmidt, R., Suglia, S.F., Tung, I., Weisenberger, D., Zhu, Y., Fry, R., 2021.

Exploring the evidence for epigenetic regulation of environmental influences on child health across generations. Commun. Biol. 4, 1–15. https://doi.org/10.1038/s42003-021-02316-6

25) Brummelte, S., Galea, L.A.M., 2010. Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. Horm. Behav. 58, 769–779. https://doi.org/10.1016/j.yhbeh.2010.07.012
26) Brunton, P.J., 2013. Effects of maternal exposure to social stress during pregnancy: consequences for mother and offspring. Reprod. Camb. Engl. 146, R175-189. https://doi.org/10.1530/REP-13-0258
27) Brunton, P.J., Russell, J.A., 2008. The expectant brain: adapting for motherhood. Nat. Rev. Neurosci. 9, 11–25. https://doi.org/10.1038/nrn2280
28) Brunton, P.J., Russell, J.A., Douglas, A.J., 2008. Adaptive responses of the maternal hypothalamic-pituitary-adrenal axis during pregnancy and lactation. J. Neuroendocrinol. 20, 764–776. https://doi.org/10.1111/j.1365-2826.2008.01735.x
29) Buffington, S.A., Viana Di Prisco, G., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli, M., 2016. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell 165, 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001
30) Butt, U.D., Lin, N., Akhter, N., Siddiqui, T., Li, S., Wu, B., 2021. Overview of the latest developments in the role of probiotics, prebiotics and synbiotics in shrimp aquaculture. Fish Shellfish Immunol. 114, 263–281. https://doi.org/10.1016/j.fsi.2021.05.003
31) Calcagnoli, F., Kreutzmann, J.C., de Boer, S.F., Althaus, M., Koolhaas, J.M., 2015. Acute and repeated intranasal oxytocin administration exerts anti-aggressive and pro-affiliative effects in male rats. Psychoneuroendocrinology, This issue includes a Special Section on Biomarkers in the Military - New Findings from Prospective Studies 51, 112–121. https://doi.org/10.1016/j.psyneuen.2014.09.019
32) Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P.M., Meaney, M.J., 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc. Natl. Acad. Sci. U. S. A. 95, 5335–5340. https://doi.org/10.1073/pnas.95.9.5335
33) Cannon, W.B., 1915. Bodily changes in pain, hunger, fear and rage: An account of recent researches into the function of emotional excitement, Bodily changes in pain, hunger, fear and rage: An account of recent researches into the function of emotional excitement. D Appleton & Company, New York, NY, US. https://doi.org/10.1037/10013-000
34) Cao-Lei, L., Dancause, K.N., Elgbeili, G., Laplante, D.P., Szyf, M., King, S., 2018. DNA methylation mediates the effect of maternal cognitive appraisal of a disaster in pregnancy on the child's C-peptide secretion in adolescence: Project Ice Storm. PloS One 13, e0192199.

https://doi.org/10.1371/journal.pone.0192199

35) Cao-Lei, L., Dancause, K.N., Elgbeili, G., Massart, R., Szyf, M., Liu, A., Laplante, D.P., King, S., 2015. DNA methylation mediates the impact of exposure to prenatal maternal stress on BMI and central adiposity in children at age 13½ years: Project Ice Storm. Epigenetics 10, 749–761. https://doi.org/10.1080/15592294.2015.1063771
36) Carter, C.S., 2014. OXYTOCIN PATHWAYS AND THE EVOLUTION OF HUMAN BEHAVIOR. https://doi.org/10.1146/annurev-psych-010213-115110
37) Carter, C.S., Altemus, M., Chrousos, G.P., 2001. Neuroendocrine and emotional changes in the post-partum period. Prog. Brain Res. 133, 241–249. https://doi.org/10.1016/s0079-6123(01)33018-2
38) Carter, C.S., Kenkel, W.M., MacLean, E.L., Wilson, S.R., Perkeybile, A.M., Yee, J.R., Ferris, C.F., Nazarloo, H.P., Porges, S.W., Davis, J.M., Connelly, J.J., Kingsbury, M.A., 2020. Is Oxytocin "Nature's Medicine"? Pharmacol. Rev. 72, 829–861. https://doi.org/10.1124/pr.120.019398
39) Castrén, E., Rantamäki, T., 2010. The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. Dev. Neurobiol. 70, 289–297. https://doi.org/10.1002/dneu.20758
40) Cevik, A., Alan, S., 2021. Are pregnancy and postpartum oxytocin level a predictive biomarker for postpartum depression? J. Obstet. Gynaecol. Res. 47, 4280–4288. https://doi.org/10.1111/jog.15023
41) Cho, I., Blaser, M.J., 2012. The Human Microbiome: at the interface of health and disease. Nat. Rev. Genet. 13, 260–270. https://doi.org/10.1038/nrg3182
42) Christiaens, I., Hegadoren, K., Olson, D.M., 2015. Adverse childhood experiences are associated with spontaneous preterm birth: a case–control study. BMC Med. 13, 124. https://doi.org/10.1186/s12916-015-0353-0
43) Codagnone, M.G., Spichak, S., O'Mahony, S.M., O'Leary, O.F., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2019. Programming Bugs: Microbiota and the Developmental Origins of Brain Health and Disease. Biol. Psychiatry 85, 150–163. https://doi.org/10.1016/j.biopsych.2018.06.014
44) Colucci-D'Amato, L., Speranza, L., Volpicelli, F., 2020. Neurotrophic Factor BDNF, Physiological Functions and Therapeutic Potential in Depression, Neurodegeneration and Brain Cancer. Int. J. Mol. Sci. 21, 7777. https://doi.org/10.3390/ijms21207777
45) Cooklin, A.R., Amir, L.H., Nguyen, C.D., Buck, M.L., Cullinane, M., Fisher, J.R.W., Donath, S.M., CASTLE Study Team, 2018. Physical health, breastfeeding problems and maternal mood in the early postpartum: a prospective cohort study. Arch. Womens Ment. Health 21,

365–374. https://doi.org/10.1007/s00737-017-0805-y

46) Coutinho, A.E., Chapman, K.E., 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol. Cell. Endocrinol. 335, 2–13. https://doi.org/10.1016/j.mce.2010.04.005

47) Cryan, J.F., Dinan, T.G., 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 13, 701–712. https://doi.org/10.1038/nrn3346

48) Dancause, K.N., Veru, F., Andersen, R.E., Laplante, D.P., King, S., 2013. Prenatal stress due to a natural disaster predicts insulin secretion in adolescence. Early Hum. Dev. 89, 773–776. https://doi.org/10.1016/j.earlhumdev.2013.06.006

49) Danhof, H.A., Lee, J., Thapa, A., Britton, R.A., Di Rienzi, S.C., 2023. Microbial stimulation of oxytocin release from the intestinal epithelium via secretin signaling. BioRxiv Prepr. Serv. Biol. 2023.03.09.531917. https://doi.org/10.1101/2023.03.09.531917

50) Darnaudéry, M., Dutriez, I., Viltart, O., Morley-Fletcher, S., Maccari, S., 2004. Stress during gestation induces lasting effects on emotional reactivity of the dam rat. Behav. Brain Res. 153, 211–216. https://doi.org/10.1016/j.bbr.2003.12.001

51) de Kloet, E.R. de, Meijer, O.C., 2019. MR/GR Signaling in the Brain during the Stress Response, in: Aldosterone-Mineralocorticoid Receptor - Cell Biology to Translational Medicine. IntechOpen. https://doi.org/10.5772/intechopen.87234

52) de Kloet, E.R., Joëls, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6, 463–475. https://doi.org/10.1038/nrn1683

53) Debiec, J., Sullivan, R.M., 2014. Intergenerational transmission of emotional trauma through amygdala-dependent mother-to-infant transfer of specific fear. Proc. Natl. Acad. Sci. U. S. A. 111, 12222–12227. https://doi.org/10.1073/pnas.1316740111

54) Dee, G., Ryznar, R., Dee, C., 2023. Epigenetic Changes Associated with Different Types of Stressors and Suicide. Cells 12, 1258. https://doi.org/10.3390/cells12091258

55) Dias, B.G., Ressler, K.J., 2014. Parental olfactory experience influences behavior and neural structure in subsequent generations. Nat. Neurosci. 17, 89–96. https://doi.org/10.1038/nn.3594

56) Dinan, T.G., Stanton, C., Cryan, J.F., 2013. Psychobiotics: A Novel Class of Psychotropic. Biol. Psychiatry, Novel Therapeutics for Depression 74, 720–726. https://doi.org/10.1016/j.biopsych.2013.05.001

57) Dominguez-Bello, M.G., De Jesus-Laboy, K.M., Shen, N., Cox, L.M., Amir, A., Gonzalez, A., Bokulich, N.A., Song, S.J., Hoashi, M., Rivera-Vinas, J.I., Mendez, K., Knight, R., Clemente, J.C., 2016. Partial restoration of the microbiota of cesarean-born infants via vaginal

microbial transfer. Nat. Med. 22, 250–253. https://doi.org/10.1038/nm.4039

58) Dooling, S.W., Sgritta, M., Wang, I.-C., Duque, A.L.R.F., Costa-Mattioli, M., 2022. The Effect of Limosilactobacillus reuteri on Social Behavior Is Independent of the Adaptive Immune System. mSystems 7, e0035822. https://doi.org/10.1128/msystems.00358-22
59) Drake, A.J., Walker, B.R., 2004. The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk. https://doi.org/10.1677/joe.0.1800001
60) Du Vigneaud, V., Ressler, C., Trippett, S., 1953. The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. J. Biol. Chem. 205, 949–957.
61) Erdman, S.E., Poutahidis, T., 2016. Microbes and Oxytocin: Benefits for Host Physiology and Behavior. Int. Rev. Neurobiol. 131, 91–126. https://doi.org/10.1016/bs.irn.2016.07.004
62) Fahmy, N.G., Yousef, H.M., Zaki, H.V., 2016. Comparative study between effect of carbetocin and oxytocin on isoflurane-induced uterine hypotonia in twin pregnancy patients undergoing cesarean section. Egypt. J. Anaesth. 32, 117–121. https://doi.org/10.1016/j.egja.2015.09.012
63) Fernandez-Twinn, D.S., Hjort, L., Novakovic, B., Ozanne, S.E., Saffery, R., 2019. Intrauterine programming of obesity and type 2 diabetes. Diabetologia 62, 1789–1801. https://doi.org/10.1007/s00125-019-4951-9
64) Flory, J.D., Bierer, L.M., Yehuda, R., 2011. Maternal Exposure to the Holocaust and Health Complaints in Offspring. Dis. Markers 30, 250470. https://doi.org/10.3233/DMA-2011-0748
65) Foltran, R.B., Diaz, S.L., 2016. BDNF isoforms: a round trip ticket between neurogenesis and serotonin? J. Neurochem. 138, 204–221. https://doi.org/10.1111/jnc.13658
66) Francis, D., Diorio, J., Liu, D., Meaney, M.J., 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286, 1155–1158. https://doi.org/10.1126/science.286.5442.1155
67) Franklin, T.B., Russig, H., Weiss, I.C., Gräff, J., Linder, N., Michalon, A., Vizi, S., Mansuy, I.M., 2010. Epigenetic transmission of the impact of early stress across generations. Biol. Psychiatry 68, 408–415. https://doi.org/10.1016/j.biopsych.2010.05.036
68) Galton, 1914. Hereditary Genius: An Inquiry into its Laws and Consequences. Nature 93, 453–453. https://doi.org/10.1038/093453b0
69) Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., Mansuy, I.M., 2014. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat. Neurosci. 17, 667–669. https://doi.org/10.1038/nn.3695
70) Gatta, E., Mairesse, J., Deruyter, L., Marrocco, J., Van Camp, G., Bouwalerh, H., Lo Guidice, J.-M., Morley-Fletcher, S., Nicoletti, F., Maccari, S., 2018. Reduced maternal behavior caused

by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance. NeuroToxicology 66, 138–149. https://doi.org/10.1016/j.neuro.2018.04.005

71) Grinevich, V., Desarménien, M.G., Chini, B., Tauber, M., Muscatelli, F., 2015. Ontogenesis of oxytocin pathways in the mammalian brain: late maturation and psychosocial disorders. Front. Neuroanat. 8. https://doi.org/10.3389/fnana.2014.00164
72) Grinevich, V., Neumann, I.D., 2021. Brain oxytocin: how puzzle stones from animal studies translate into psychiatry. Mol. Psychiatry 26, 265–279. https://doi.org/10.1038/s41380-020-0802-9
73) Grundwald, N.J., Brunton, P.J., 2015. Prenatal stress programs neuroendocrine stress responses and affective behaviors in second generation rats in a sex-dependent manner. Psychoneuroendocrinology 62, 204–216. https://doi.org/10.1016/j.psyneuen.2015.08.010
74) Guidotti, A., Auta, J., Davis, J.M., Di-Giorgi-Gerevini, V., Dwivedi, Y., Grayson, D.R., Impagnatiello, F., Pandey, G., Pesold, C., Sharma, R., Uzunov, D., Costa, E., 2000. Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. Arch. Gen. Psychiatry 57, 1061–1069. https://doi.org/10.1001/archpsyc.57.11.1061
75) Hamdan, A., Tamim, H., 2012. The relationship between postpartum depression and breastfeeding. Int. J. Psychiatry Med. 43, 243–259. https://doi.org/10.2190/PM.43.3.d
76) Harlow, H.F., Zimmermann, R.R., 1959. Affectional responses in the infant monkey; orphaned baby monkeys develop a strong and persistent attachment to inanimate surrogate mothers. Science 130, 421–432. https://doi.org/10.1126/science.130.3373.421
77) Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E., Lumey, L.H., 2008. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc. Natl. Acad. Sci. U. S. A. 105, 17046–17049. https://doi.org/10.1073/pnas.0806560105
78) Heinrichs, M., Meinlschmidt, G., Neumann, I., Wagner, S., Kirschbaum, C., Ehlert, U., Hellhammer, D.H., 2001. Effects of suckling on hypothalamic-pituitary-adrenal axis responses to psychosocial stress in postpartum lactating women. J. Clin. Endocrinol. Metab. 86, 4798–4804. https://doi.org/10.1210/jcem.86.10.7919
79) Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr. Physiol. 6, 603–621. https://doi.org/10.1002/cphy.c150015
80) HUANG, S., LI, G., PAN, Y., SONG, M., ZHAO, J., WAN, X., KREBS, C.J., WANG, Z., HAN, W., ZHANG, Z., 2021. Density‐induced social stress alters oxytocin and vasopressin

activities in the brain of a small rodent species. Integr. Zool. 16, 149–159. https://doi.org/10.1111/1749-4877.12467

81) Huang, T., Larsen, K.T., Ried-Larsen, M., Møller, N.C., Andersen, L.B., 2014. The effects of physical activity and exercise on brain-derived neurotrophic factor in healthy humans: A review. Scand. J. Med. Sci. Sports 24, 1–10. https://doi.org/10.1111/sms.12069
82) Ivell, R., Ludwig, M., Tribe, R.M., Anand-Ivell, R., 2018. Oxytocin, in: Encyclopedia of Reproduction. Elsevier, pp. 597–606. https://doi.org/10.1016/B978-0-12-801238-3.64691-3
83) Jašarević, E., Rodgers, A.B., Bale, T.L., 2014. A novel role for maternal stress and microbial transmission in early life programming and neurodevelopment. Neurobiol. Stress 1, 81–88. https://doi.org/10.1016/j.ynstr.2014.10.005
84) Jensen, P., 2013. Transgenerational epigenetic effects on animal behaviour. Prog. Biophys. Mol. Biol. 113, 447–454. https://doi.org/10.1016/j.pbiomolbio.2013.01.001
85) Jiang, J., Yang, M., Tian, M., Chen, Z., Xiao, L., Gong, Y., 2023. Intertwined associations between oxytocin, immune system and major depressive disorder. Biomed. Pharmacother. 163, 114852. https://doi.org/10.1016/j.biopha.2023.114852
86) Jin, X.-H., Li, D., Li, X., 2019. Carbetocin vs oxytocin for prevention of postpartum hemorrhage after vaginal delivery. Medicine (Baltimore) 98, e17911. https://doi.org/10.1097/MD.0000000000017911
87) Jung, I.-H., Jung, M.-A., Kim, E.-J., Han, M.J., Kim, D.-H., 2012. Lactobacillus pentosus var. plantarum C29 protects scopolamine-induced memory deficit in mice. J. Appl. Microbiol. 113, 1498–1506. https://doi.org/10.1111/j.1365-2672.2012.05437.x
88) Jurek, B., Neumann, I.D., 2018. The Oxytocin Receptor: From Intracellular Signaling to Behavior. Physiol. Rev. 98, 1805–1908. https://doi.org/10.1152/physrev.00031.2017
89) Kartry, A.L., Keith-Lee, P., Morton, W.D., 1964. PLANARIA: MEMORY TRANSFER THROUGH CANNIBALISM REEXAMINED. Science 146, 274–275. https://doi.org/10.1126/science.146.3641.274
90) Kelly, J.R., Borre, Y., O' Brien, C., Patterson, E., El Aidy, S., Deane, J., Kennedy, P.J., Beers, S., Scott, K., Moloney, G., Hoban, A.E., Scott, L., Fitzgerald, P., Ross, P., Stanton, C., Clarke, G., Cryan, J.F., Dinan, T.G., 2016. Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat. J. Psychiatr. Res. 82, 109–118. https://doi.org/10.1016/j.jpsychires.2016.07.019
91) Kenkel, W.M., Perkeybile, A.-M., Yee, J.R., Pournajafi-Nazarloo, H., Lillard, T.S., Ferguson, E.F., Wroblewski, K.L., Ferris, C.F., Carter, C.S., Connelly, J.J., 2019. Behavioral and epigenetic consequences of oxytocin treatment at birth. Sci. Adv. 5, eaav2244. https://doi.org/10.1126/sciadv.aav2244

92) Kenkel, W.M., Yee, J.R., Carter, C.S., 2014. Is oxytocin a maternal-foetal signalling molecule at birth? Implications for development. J. Neuroendocrinol. 26, 739–749. https://doi.org/10.1111/jne.12186

93) Kim, T.Y., Kim, S.J., Chung, H.G., Choi, J.H., Kim, S.H., Kang, J.I., 2017. Epigenetic alterations of the BDNF gene in combat-related post-traumatic stress disorder. Acta Psychiatr. Scand. 135, 170–179. https://doi.org/10.1111/acps.12675

94) Kimmel, M., Clive, M., Gispen, F., Guintivano, J., Brown, T., Cox, O., Beckmann, M.W., Kornhuber, J., Fasching, P.A., Osborne, L.M., Binder, E., Payne, J.L., Kaminsky, Z., 2016. Oxytocin receptor DNA methylation in postpartum depression. Psychoneuroendocrinology 69, 150–160. https://doi.org/10.1016/j.psyneuen.2016.04.008

95) King, S., Laplante, D.P., 2005. The effects of prenatal maternal stress on children's cognitive development: Project Ice Storm. Stress 8, 35–45. https://doi.org/10.1080/10253890500108391

96) Klampfl, S.M., Schramm, M.M., Gaßner, B.M., Hübner, K., Seasholtz, A.F., Brunton, P.J., Bayerl, D.S., Bosch, O.J., 2018. Maternal stress and the MPOA: Activation of CRF receptor 1 impairs maternal behavior and triggers local oxytocin release in lactating rats. Neuropharmacology 133, 440–450. https://doi.org/10.1016/j.neuropharm.2018.02.019

97) Klengel, T., Dias, B.G., Ressler, K.J., 2016. Models of Intergenerational and Transgenerational Transmission of Risk for Psychopathology in Mice. Neuropsychopharmacology 41, 219–231. https://doi.org/10.1038/npp.2015.249

98) Koehl, M., Barbazanges, A., Le Moal, M., Maccari, S., 1997. Prenatal stress induces a phase advance of circadian corticosterone rhythm in adult rats which is prevented by postnatal stress. Brain Res. 759, 317–320. https://doi.org/10.1016/s0006-8993(97)00394-6

99) Kowiański, P., Lietzau, G., Czuba, E., Waśkow, M., Steliga, A., Moryś, J., 2018. BDNF: A Key Factor with Multipotent Impact on Brain Signaling and Synaptic Plasticity. Cell. Mol. Neurobiol. 38, 579–593. https://doi.org/10.1007/s10571-017-0510-4

100) Krol, K.M., Grossmann, T., 2018. Psychological effects of breastfeeding on children and mothers. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 61, 977–985. https://doi.org/10.1007/s00103-018-2769-0

101) Laloux, C., Mairesse, J., Van Camp, G., Giovine, A., Branchi, I., Bouret, S., Morley-Fletcher, S., Bergonzelli, G., Malagodi, M., Gradini, R., Nicoletti, F., Darnaudéry, M., Maccari, S., 2012. Anxiety-like behaviour and associated neurochemical and endocrinological alterations in male pups exposed to prenatal stress. Psychoneuroendocrinology 37, 1646–1658. https://doi.org/10.1016/j.psyneuen.2012.02.010

102) Langarizadeh, M.A., Ranjbar Tavakoli, M., Abiri, A., Ghasempour, A., Rezaei, M., Ameri, A., 2021. A review on function and side effects of systemic corticosteroids used in

high-grade COVID-19 to prevent cytokine storms. EXCLI J. 20, 339–365. https://doi.org/10.17179/excli2020-3196

103) Lapp, H.E., Salazar, M.G., Champagne, F.A., 2023. Automated maternal behavior during early life in rodents (AMBER) pipeline. Sci. Rep. 13, 18277. https://doi.org/10.1038/s41598-023-45495-4

104) Lee, H.J., Hong, J.K., Kim, J.-K., Kim, D.-H., Jang, S.W., Han, S.-W., Yoon, I.-Y., 2021. Effects of Probiotic NVP-1704 on Mental Health and Sleep in Healthy Adults: An 8-Week Randomized, Double-Blind, Placebo-Controlled Trial. Nutrients 13, 2660. https://doi.org/10.3390/nu13082660

105) Lee, M.R., Shnitko, T.A., Blue, S.W., Kaucher, A.V., Winchell, A.J., Erikson, D.W., Grant, K.A., Leggio, L., 2020. Labeled oxytocin administered via the intranasal route reaches the brain in rhesus macaques. Nat. Commun. 11, 2783. https://doi.org/10.1038/s41467-020-15942-1

106) Lee, Y., Ba, Z., Roberts, R.F., Rogers, C.J., Fleming, J.A., Meng, H., Furumoto, E.J., Kris-Etherton, P.M., 2017. Effects of Bifidobacterium animalis subsp. lactis BB-12® on the lipid/lipoprotein profile and short chain fatty acids in healthy young adults: a randomized controlled trial. Nutr. J. 16, 39. https://doi.org/10.1186/s12937-017-0261-6

107) Lee, Y., Kim, K.-H., Lee, B.-H., Kim, Y.-K., 2021. Plasma level of brain-derived neurotrophic factor (BDNF) in patients with postpartum depression. Prog. Neuropsychopharmacol. Biol. Psychiatry 109, 110245. https://doi.org/10.1016/j.pnpbp.2021.110245

108) Lesage, J., Del-Favero, F., Leonhardt, M., Louvart, H., Maccari, S., Vieau, D., Darnaudery, M., 2004. Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. J. Endocrinol. 181, 291–296. https://doi.org/10.1677/joe.0.1810291

109) Lester, B.M., Conradt, E., LaGasse, L.L., Tronick, E.Z., Padbury, J.F., Marsit, C.J., 2018. Epigenetic Programming by Maternal Behavior in the Human Infant. Pediatrics 142, e20171890. https://doi.org/10.1542/peds.2017-1890

110) Levine, S., 1957. Infantile experience and resistance to physiological stress. Science 126, 405. https://doi.org/10.1126/science.126.3270.405

111) Levine, S.Z., Levav, I., Pugachova, I., Yoffe, R., Becher, Y., 2016. Transgenerational effects of genocide exposure on the risk and course of schizophrenia: A population-based study. Schizophr. Res. 176, 540–545. https://doi.org/10.1016/j.schres.2016.06.019

112) Li, H., Lin, L.-Y., Zhang, Y., Lim, Y., Rahman, M., Beck, A., Al-Hawwas, M., Feng, S., Bobrovskaya, L., Zhou, X.-F., 2020. Pro-BDNF Knockout Causes Abnormal Motor

Behaviours and Early Death in Mice. Neuroscience 438, 145–157. https://doi.org/10.1016/j.neuroscience.2020.05.007

113) Li, X., Laplante, D.P., Elgbeili, G., King, S., 2023. Preconception and prenatal maternal stress are associated with broad autism phenotype in young adults: Project Ice Storm. J. Dev. Orig. Health Dis. 14, 481–489. https://doi.org/10.1017/S2040174423000156

114) Liao, P.-Y., Chiu, Y.-M., Yu, J.-H., Chen, S.-K., 2020. Mapping Central Projection of Oxytocin Neurons in Unmated Mice Using Cre and Alkaline Phosphatase Reporter. Front. Neuroanat. 14. https://doi.org/10.3389/fnana.2020.559402

115) Logue, M.W., Miller, M.W., Wolf, E.J., Huber, B.R., Morrison, F.G., Zhou, Z., Zheng, Y., Smith, A.K., Daskalakis, N.P., Ratanatharathorn, A., Uddin, M., Nievergelt, C.M., Ashley-Koch, A.E., Baker, D.G., Beckham, J.C., Garrett, M.E., Boks, M.P., Geuze, E., Grant, G.A., Hauser, M.A., Kessler, R.C., Kimbrel, N.A., Maihofer, A.X., Marx, C.E., Qin, X.-J., Risbrough, V.B., Rutten, B.P.F., Stein, M.B., Ursano, R.J., Vermetten, E., Vinkers, C.H., Ware, E.B., Stone, A., Schichman, S.A., McGlinchey, R.E., Milberg, W.P., Hayes, J.P., Verfaellie, M., Traumatic Stress Brain Study Group, 2020. An epigenome-wide association study of posttraumatic stress disorder in US veterans implicates several new DNA methylation loci. Clin. Epigenetics 12, 46. https://doi.org/10.1186/s13148-020-0820-0

116) Lopes, N.A., Ambeskovic, M., King, S.E., Faraji, J., Soltanpour, N., Falkenberg, E.A., Scheidl, T., Patel, M., Fang, X., Metz, G.A.S., Olson, D.M., 2023. Environmental Enrichment Promotes Transgenerational Programming of Uterine Inflammatory and Stress Markers Comparable to Gestational Chronic Variable Stress. Int. J. Mol. Sci. 24, 3734. https://doi.org/10.3390/ijms24043734

117) Lumey, L.H., Stein, A.D., Susser, E., 2011. Prenatal Famine and Adult Health. Annu. Rev. Public Health 32, 10.1146/annurev-publhealth-031210–101230. https://doi.org/10.1146/annurev-publhealth-031210-101230

118) Maccari, S., Darnaudery, M., Morley-Fletcher, S., Zuena, A.R., Cinque, C., Van Reeth, O., 2003. Prenatal stress and long-term consequences: implications of glucocorticoid hormones. Neurosci. Biobehav. Rev. 27, 119–127. https://doi.org/10.1016/s0149-7634(03)00014-9

119) Maccari, S., Krugers, H.J., Morley-Fletcher, S., Szyf, M., Brunton, P.J., 2014. The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. J. Neuroendocrinol. 26, 707–723. https://doi.org/10.1111/jne.12175

120) Maccari, S., Piazza, P.V., Kabbaj, M., Barbazanges, A., Simon, H., Le Moal, M., 1995. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J. Neurosci. Off. J. Soc. Neurosci. 15, 110–116.

121) Maccari, S., Polese, D., Reynaert, M.-L., Amici, T., Morley-Fletcher, S., Fagioli, F., 2017. Early-life experiences and the development of adult diseases with a focus on mental illness: The Human Birth Theory. Neuroscience 342, 232–251. https://doi.org/10.1016/j.neuroscience.2016.05.042

122) Mairesse, J., Lesage, J., Breton, C., Bréant, B., Hahn, T., Darnaudéry, M., Dickson, S.L., Seckl, J., Blondeau, B., Vieau, D., Maccari, S., Viltart, O., 2007. Maternal stress alters endocrine function of the feto-placental unit in rats. Am. J. Physiol. Endocrinol. Metab. 292, E1526-1533. https://doi.org/10.1152/ajpendo.00574.2006

123) Marlin, B.J., Mitre, M., D'amour, J.A., Chao, M.V., Froemke, R.C., 2015. Oxytocin Enables Maternal Behavior by Balancing Cortical Inhibition. Nature 520, 499–504. https://doi.org/10.1038/nature14402

124) Marrocco, J., Mairesse, J., Ngomba, R.T., Silletti, V., Van Camp, G., Bouwalerh, H., Summa, M., Pittaluga, A., Nicoletti, F., Maccari, S., Morley-Fletcher, S., 2012. Anxiety-Like Behavior of Prenatally Stressed Rats Is Associated with a Selective Reduction of Glutamate Release in the Ventral Hippocampus. J. Neurosci. 32, 17143–17154. https://doi.org/10.1523/JNEUROSCI.1040-12.2012

125) Matrisciano, F., Tueting, P., Dalal, I., Kadriu, B., Grayson, D.R., Davis, J.M., Nicoletti, F., Guidotti, A., 2013. EPIGENETIC MODIFICATIONS OF GABAERGIC INTERNEURONS ARE ASSOCIATED WITH THE SCHIZOPHRENIA-LIKE PHENOTYPE INDUCED BY PRENATAL STRESS IN MICE. Neuropharmacology 68, 184–194. https://doi.org/10.1016/j.neuropharm.2012.04.013

126) Matsuzaki, M., Matsushita, H., Tomizawa, K., Matsui, H., 2012. Oxytocin: A therapeutic target for mental disorders. J. Physiol. Sci. 62. https://doi.org/10.1007/s12576-012-0232-9

127) Mauro, C.S.I., Guergoletto, K.B., Garcia, S., 2016. Development of Blueberry and Carrot Juice Blend Fermented by Lactobacillus reuteri LR92. Beverages 2, 37. https://doi.org/10.3390/beverages2040037

128) Maynard, K.R., Hobbs, J.W., Phan, B.N., Gupta, A., Rajpurohit, S., Williams, C., Rajpurohit, A., Shin, J.H., Jaffe, A.E., Martinowich, K., 2018. BDNF-TrkB signaling in oxytocin neurons contributes to maternal behavior. eLife 7, e33676. https://doi.org/10.7554/eLife.33676

129) McEwen, B.S., 2012. The Ever-Changing Brain: Cellular and Molecular Mechanisms for the Effects of Stressful Experiences. Dev. Neurobiol. 72, 878–890. https://doi.org/10.1002/dneu.20968

130) Meaney, M.J., 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu. Rev. Neurosci. 24, 1161–1192.

https://doi.org/10.1146/annurev.neuro.24.1.1161

131) Meisenberg, G., 1981. Short-term behavioral effects of posterior pituitary peptides in mice. Peptides 2, 1–8. https://doi.org/10.1016/S0196-9781(81)80003-4

132) Mennickent, D., Rodríguez, A., Opazo, Ma.C., Riedel, C.A., Castro, E., Eriz-Salinas, A., Appel-Rubio, J., Aguayo, C., Damiano, A.E., Guzmán-Gutiérrez, E., Araya, J., 2023. Machine learning applied in maternal and fetal health: a narrative review focused on pregnancy diseases and complications. Front. Endocrinol. 14, 1130139. https://doi.org/10.3389/fendo.2023.1130139

133) Merchak, A.R., Wachamo, S., Brown, L.C., Thakur, A., Moreau, B., Brown, R.M., Rivet-Noor, C.R., Raghavan, T., Gaultier, A., 2024. *Lactobacillus* from the Altered Schaedler Flora maintain IFNγ homeostasis to promote behavioral stress resilience. Brain. Behav. Immun. 115, 458–469. https://doi.org/10.1016/j.bbi.2023.11.001

134) Metchnikoff, E., 1908. The prolongation of life : optimistic studies / by Élie Metchnikoff ; English translation edited by P. Chalmers Mitchell. [WWW Document]. Wellcome Collect. URL https://wellcomecollection.org/works/a5439955 (accessed 6.15.24).

135) Miller, G., 2010. Epigenetics. The seductive allure of behavioral epigenetics. Science 329, 24–27. https://doi.org/10.1126/science.329.5987.24

136) Mitre, M., Kranz, T.M., Marlin, B.J., Schiavo, J.K., Erdjument-Bromage, H., Zhang, X., Minder, J., Neubert, T.A., Hackett, T.A., Chao, M.V., Froemke, R.C., 2017. Sex-Specific Differences in Oxytocin Receptor Expression and Function for Parental Behavior. Gend. Genome 1, 1–25. https://doi.org/10.1089/gg.2017.0017

137) Mitre, M., Marlin, B.J., Schiavo, J.K., Morina, E., Norden, S.E., Hackett, T.A., Aoki, C.J., Chao, M.V., Froemke, R.C., 2016. A Distributed Network for Social Cognition Enriched for Oxytocin Receptors. J. Neurosci. 36, 2517–2535. https://doi.org/10.1523/JNEUROSCI.2409-15.2016

138) Mitre, M., Saadipour, K., Williams, K., Khatri, L., Froemke, R.C., Chao, M.V., 2022. Transactivation of TrkB Receptors by Oxytocin and Its G Protein-Coupled Receptor. Front. Mol. Neurosci. 15, 891537. https://doi.org/10.3389/fnmol.2022.891537

139) Moisiadis, V.G., Constantinof, A., Kostaki, A., Szyf, M., Matthews, S.G., 2017. Prenatal Glucocorticoid Exposure Modifies Endocrine Function and Behaviour for 3 Generations Following Maternal and Paternal Transmission | Scientific Reports. Sci. Rep. 7, 11814. https://doi.org/10.1038/s41598-017-11635-w

140) Morley-Fletcher, S., Darnaudery, M., Koehl, M., Casolini, P., Van Reeth, O., Maccari, S., 2003. Prenatal stress in rats predicts immobility behavior in the forced swim test: Effects of a chronic treatment with tianeptine. Brain Res. 989, 246–251.

https://doi.org/10.1016/S0006-8993(03)03293-1

141) Morley-Fletcher, S., Gaetano, A., Gao, V., Gatta, E., Van Camp, G., Bouwalerh, H., Thomas, P., Nicoletti, F., Maccari, S., 2024. Postpartum Oxytocin Treatment via the Mother Reprograms Long-Term Behavioral Disorders Induced by Early Life Stress on the Plasma and Brain Metabolome in the Rat. Int. J. Mol. Sci. 25, 3014. https://doi.org/10.3390/ijms25053014

142) Morley-Fletcher, S., Mairesse, J., Soumier, A., Banasr, M., Fagioli, F., Gabriel, C., Mocaer, E., Daszuta, A., McEwen, B., Nicoletti, F., Maccari, S., 2011. Chronic agomelatine treatment corrects behavioral, cellular, and biochemical abnormalities induced by prenatal stress in rats. Psychopharmacology (Berl.) 217, 301–313. https://doi.org/10.1007/s00213-011-2280-x

143) Morley-Fletcher, S., Zuena, A.R., Mairesse, J., Gatta, E., Van Camp, G., Bouwalerh, H., Riozzi, B., Battaglia, G., Pittaluga, A., Olivero, G., Mocaer, E., Bretin, S., Nicoletti, F., Maccari, S., 2018. The reduction in glutamate release is predictive of cognitive and emotional alterations that are corrected by the positive modulator of AMPA receptors S 47445 in perinatal stressed rats. Neuropharmacology 135, 284–296. https://doi.org/10.1016/j.neuropharm.2018.03.018

144) Mowla, S.J., Farhadi, H.F., Pareek, S., Atwal, J.K., Morris, S.J., Seidah, N.G., Murphy, R.A., 2001. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J. Biol. Chem. 276, 12660–12666. https://doi.org/10.1074/jbc.M008104200

145) Mueller, B.R., Bale, T.L., 2008. Sex-specific programming of offspring emotionality after stress early in pregnancy. J. Neurosci. Off. J. Soc. Neurosci. 28, 9055–9065. https://doi.org/10.1523/JNEUROSCI.1424-08.2008

146) Murakami, G., Hunter, R.G., Fontaine, C., Ribeiro, A., Pfaff, D., 2011. Relationships among estrogen receptor, oxytocin and vasopressin gene expression and social interaction in male mice. Eur. J. Neurosci. 34, 469–477. https://doi.org/10.1111/j.1460-9568.2011.07761.x

147) Mychasiuk, R., Schmold, N., Ilnytskyy, S., Kovalchuk, O., Kolb, B., Gibb, R., 2011. Prenatal bystander stress alters brain, behavior, and the epigenome of developing rat offspring. Dev. Neurosci. 33, 159–169. https://doi.org/10.1159/000330034

148) Nagasawa, M., Okabe, S., Mogi, K., Kikusui, T., 2012. Oxytocin and mutual communication in mother-infant bonding. Front. Hum. Neurosci. 6, 31. https://doi.org/10.3389/fnhum.2012.00031

149) Neufeld, Kang, N., Bienenstock, J., Foster, J.A., 2011a. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol. Motil. 23, 255–264, e119. https://doi.org/10.1111/j.1365-2982.2010.01620.x

150) Neufeld, Kang, N., Bienenstock, J., Foster, J.A., 2011b. Effects of intestinal microbiota on anxiety-like behavior. Commun. Integr. Biol. 4, 492–494. https://doi.org/10.4161/cib.4.4.15702

151) Notaras, M., van den Buuse, M., 2019. Brain-Derived Neurotrophic Factor (BDNF): Novel Insights into Regulation and Genetic Variation. The Neuroscientist 25, 434–454. https://doi.org/10.1177/1073858418810142

152) Orso, R., Creutzberg, K.C., Wearick-Silva, L.E., Wendt Viola, T., Tractenberg, S.G., Benetti, F., Grassi-Oliveira, R., 2019. How Early Life Stress Impact Maternal Care: A Systematic Review of Rodent Studies. Front. Behav. Neurosci. 13.

153) O'Sullivan, E., Grenham, S., Barrett, E., Fitzgerald, P., Stanton, C., Ross, R.P., Cryan, J.F., Quigley, E.M., Dinan, T.G., 2011. BDNF Expression in the Hippocampus of Maternally Separated Rats: Does Bifidobacterium Breve 6330 Alter BDNF Levels? Gastroenterology, 2011 DDW Abstract Supplement 140, S-850. https://doi.org/10.1016/S0016-5085(11)63528-2

154) Painter, R.C., Osmond, C., Gluckman, P., Hanson, M., Phillips, D.I.W., Roseboom, T.J., 2008. Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. BJOG Int. J. Obstet. Gynaecol. 115, 1243–1249. https://doi.org/10.1111/j.1471-0528.2008.01822.x

155) Papadopoulos, A.S., Cleare, A.J., 2012. Hypothalamic–pituitary–adrenal axis dysfunction in chronic fatigue syndrome. Nat. Rev. Endocrinol. 8, 22–32. https://doi.org/10.1038/nrendo.2011.153

156) Park, E.S., Freeborn, J., Venna, V.R., Roos, S., Rhoads, J.M., Liu, Y., 2021. Lactobacillus reuteri effects on maternal separation stress in newborn mice. Pediatr. Res. 90, 980–988. https://doi.org/10.1038/s41390-021-01374-0

157) Patterson, E., Ryan, P.M., Wiley, N., Carafa, I., Sherwin, E., Moloney, G., Franciosi, E., Mandal, R., Wishart, D.S., Tuohy, K., Ross, R.P., Cryan, J.F., Dinan, T.G., Stanton, C., 2019. Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome. Sci. Rep. 9, 16323. https://doi.org/10.1038/s41598-019-51781-x

158) Pedersen, C.A., Gibson, C.M., Rau, S.W., Salimi, K., Smedley, K.L., Casey, R.L., Leserman, J., Jarskog, L.F., Penn, D.L., 2011. Intranasal oxytocin reduces psychotic symptoms and improves Theory of Mind and social perception in schizophrenia. Schizophr. Res. 132, 50–53. https://doi.org/10.1016/j.schres.2011.07.027

159) Pinho, G.M., Ortiz-Ross, X., Reese, A.N., Blumstein, D.T., 2019. Correlates of maternal glucocorticoid levels in a socially flexible rodent. Horm. Behav. 116, 104577. https://doi.org/10.1016/j.yhbeh.2019.104577

160) Pruunsild, P., Kazantseva1, A., Aid, T., Palm, K., Timmusk, T., 2007. Dissecting the human BDNF locus: Bidirectional transcription, complex splicing, and multiple promoters. Genomics 90, 397–406. https://doi.org/10.1016/j.ygeno.2007.05.004

161) Pundir, S., Mäkelä, J., Nuora, A., Junttila, N., Wall, C.R., Linderborg, K., Cameron-Smith, D., Lagström, H., 2019. Maternal influences on the glucocorticoid concentrations of human milk: The STEPS study. Clin. Nutr. Edinb. Scotl. 38, 1913–1920. https://doi.org/10.1016/j.clnu.2018.06.980

162) Quintana, D.S., Guastella, A.J., 2020. An Allostatic Theory of Oxytocin. Trends Cogn. Sci. 24, 515–528. https://doi.org/10.1016/j.tics.2020.03.008

163) Quintana, D.S., Lischke, A., Grace, S., Scheele, D., Ma, Y., Becker, B., 2021. Advances in the field of intranasal oxytocin research: lessons learned and future directions for clinical research. Mol. Psychiatry 26, 80–91. https://doi.org/10.1038/s41380-020-00864-7

164) Rakoff, V., 1966. A long term effect of the concentration camp experience.

165) Ramo-Fernández, L., Gumpp, A.M., Boeck, C., Krause, S., Bach, A.M., Waller, C., Kolassa, I.-T., Karabatsiakis, A., 2021. Associations between childhood maltreatment and DNA methylation of the oxytocin receptor gene in immune cells of mother–newborn dyads. Transl. Psychiatry 11, 449. https://doi.org/10.1038/s41398-021-01546-w

166) Ravelli, A.C., van der Meulen, J.H., Michels, R.P., Osmond, C., Barker, D.J., Hales, C.N., Bleker, O.P., 1998. Glucose tolerance in adults after prenatal exposure to famine. Lancet Lond. Engl. 351, 173–177. https://doi.org/10.1016/s0140-6736(97)07244-9

167) Riem, M.M.E., Bakermans-Kranenburg, M.J., Pieper, S., Tops, M., Boksem, M.A.S., Vermeiren, R.R.J.M., van Ijzendoorn, M.H., Rombouts, S.A.R.B., 2011. Oxytocin modulates amygdala, insula, and inferior frontal gyrus responses to infant crying: a randomized controlled trial. Biol. Psychiatry 70, 291–297. https://doi.org/10.1016/j.biopsych.2011.02.006

168) Riem, M.M.E., Bakermans-Kranenburg, M.J., van IJzendoorn, M.H., 2016. Intranasal administration of oxytocin modulates behavioral and amygdala responses to infant crying in females with insecure attachment representations. Attach. Hum. Dev. 18, 213–234. https://doi.org/10.1080/14616734.2016.1149872

169) Riem, M.M.E., Kunst, L.E., Bekker, M.H.J., Fallon, M., Kupper, N., 2020. Intranasal oxytocin enhances stress-protective effects of social support in women with negative childhood experiences during a virtual Trier Social Stress Test. Psychoneuroendocrinology 111, 104482. https://doi.org/10.1016/j.psyneuen.2019.104482

170) Rilling, M., 1996. The mystery of the vanished citations: James McConnell's forgotten 1960s quest for planarian learning, a biochemical engram, and celebrity. Am. Psychol. 51, 589–598. https://doi.org/10.1037/0003-066X.51.6.589

171) Robinson, C., Schumann, R., Zhang, P., Young, R.C., 2003. Oxytocin-induced desensitization of the oxytocin receptor. Am. J. Obstet. Gynecol. 188, 497–502. https://doi.org/10.1067/mob.2003.22

172) Rochette, L., Dogon, G., Vergely, C., 2023. Stress: Eight Decades after Its Definition by Hans Selye: "Stress Is the Spice of Life." Brain Sci. 13. https://doi.org/10.3390/brainsci13020310

173) Rodgers, A.B., Morgan, C.P., Leu, N.A., Bale, T.L., 2015. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. Proc. Natl. Acad. Sci. 112, 13699–13704. https://doi.org/10.1073/pnas.1508347112

174) Roseboom, T.J., Painter, R.C., van Abeelen, A.F.M., Veenendaal, M.V.E., de Rooij, S.R., 2011. Hungry in the womb: what are the consequences? Lessons from the Dutch famine. Maturitas 70, 141–145. https://doi.org/10.1016/j.maturitas.2011.06.017

175) Rosen-Carole, C.B., Greenman, S., Wang, H., Sonawane, S., Misra, R., O'Connor, T., Järvinen, K., D'Angio, C., Young, B.E., 2024. Association between maternal stress and premature milk cortisol, milk IgA, and infant health: a cohort study. Front. Nutr. 11. https://doi.org/10.3389/fnut.2024.1270523

176) Rösler, A., White, P.C., 1993. Mutations in human 11 beta-hydroxylase genes: 11 beta-hydroxylase deficiency in Jews of Morocco and corticosterone methyl-oxidase II deficiency in Jews of Iran. J. Steroid Biochem. Mol. Biol. 45, 99–106. https://doi.org/10.1016/0960-0760(93)90128-j

177) Roth, T.L., Lubin, F.D., Funk, A.J., Sweatt, J.D., 2009. LASTING EPIGENETIC INFLUENCE OF EARLY-LIFE ADVERSITY ON THE BDNF GENE. Biol. Psychiatry 65, 760–769. https://doi.org/10.1016/j.biopsych.2008.11.028

178) Russell, J.A., Douglas, A.J., 2003. Oxytocin, in: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones. Academic Press, New York, pp. 110–122. https://doi.org/10.1016/B0-12-341103-3/00228-X

179) Sanson, A., Bosch, O.J., 2022. Dysfunctions of brain oxytocin signaling: Implications for poor mothering. Neuropharmacology 211, 109049. https://doi.org/10.1016/j.neuropharm.2022.109049

180) Seckl, J.R., Holmes, M.C., 2007. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal "programming" of adult pathophysiology. Nat. Clin. Pract. Endocrinol. Metab. 3, 479–488. https://doi.org/10.1038/ncpendmet0515

181) Selhub, E.M., Logan, A.C., Bested, A.C., 2014. Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. J. Physiol. Anthropol. 33, 2. https://doi.org/10.1186/1880-6805-33-2

182) Selye, H., 1976. Stress without Distress, in: Serban, G. (Ed.), Psychopathology of Human Adaptation. Springer US, Boston, MA, pp. 137–146. https://doi.org/10.1007/978-1-4684-2238-2_9

183) Selye, H., 1952. The Physiology and Pathology of Exposure to Stress. A Treatise Based on the Concept of the General Adaptation Syndrome and the Diseases of Adaptation. Indian Med. Gaz. 87, 431.

184) Selye, H., 1936. A Syndrome produced by Diverse Nocuous Agents. Nature 138, 32–32. https://doi.org/10.1038/138032a0

185) Senaldi, L., Smith-Raska, M., 2020. Evidence for germline non-genetic inheritance of human phenotypes and diseases. Clin. Epigenetics 12, 136. https://doi.org/10.1186/s13148-020-00929-y

186) Seth, S., Lewis, A.J., Galbally, M., 2016. Perinatal maternal depression and cortisol function in pregnancy and the postpartum period: a systematic literature review. BMC Pregnancy Childbirth 16, 124. https://doi.org/10.1186/s12884-016-0915-y

187) Sgritta, M., Dooling, S.W., Buffington, S.A., Momin, E.N., Francis, M.B., Britton, R.A., Costa-Mattioli, M., 2019. Mechanisms underlying microbial-mediated changes in social behavior in mouse models of Autism Spectrum Disorder. Neuron 101, 246-259.e6. https://doi.org/10.1016/j.neuron.2018.11.018

188) Skinner, M.K., 2008. What is an epigenetic transgenerational phenotype? F3 or F2. Reprod. Toxicol. Elmsford N 25, 2–6. https://doi.org/10.1016/j.reprotox.2007.09.001

189) Skinner, M.K., Manikkam, M., Guerrero-Bosagna, C., 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. Trends Endocrinol. Metab. TEM 21, 214–222. https://doi.org/10.1016/j.tem.2009.12.007

190) Skvortsova, K., Iovino, N., Bogdanović, O., 2018. Functions and mechanisms of epigenetic inheritance in animals. Nat. Rev. Mol. Cell Biol. 19, 774–790. https://doi.org/10.1038/s41580-018-0074-2

191) Slattery, D.A., Neumann, I.D., 2008. No stress please! Mechanisms of stress hyporesponsiveness of the maternal brain. J. Physiol. 586, 377–385. https://doi.org/10.1113/jphysiol.2007.145896

192) Smith, M.P., Ayad, V.J., Mundell, S.J., McArdle, C.A., Kelly, E., López Bernal, A., 2006. Internalization and Desensitization of the Oxytocin Receptor Is Inhibited by Dynamin and Clathrin Mutants in Human Embryonic Kidney 293 Cells. Mol. Endocrinol. 20, 379–388. https://doi.org/10.1210/me.2005-0031

193) Sparén, P., Vågerö, D., Shestov, D.B., Plavinskaja, S., Parfenova, N., Hoptiar, V., Paturot, D., Galanti, M.R., 2004. Long term mortality after severe starvation during the siege of

Leningrad: prospective cohort study. BMJ 328, 11. https://doi.org/10.1136/bmj.37942.603970.9A

194) Stanner, S.A., Bulmer, K., Andrès, C., Lantseva, O.E., Borodina, V., Poteen, V.V., Yudkin, J.S., 1997. Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study. BMJ 315, 1342–1348. https://doi.org/10.1136/bmj.315.7119.1342

195) Stein, A.D., Zybert, P.A., van de Bor, M., Lumey, L.H., 2004. Intrauterine famine exposure and body proportions at birth: the Dutch Hunger Winter. Int. J. Epidemiol. 33, 831–836. https://doi.org/10.1093/ije/dyh083

196) Stenz, L., Zewdie, S., Laforge-Escarra, T., Prados, J., La Harpe, R., Dayer, A., Paoloni-Giacobino, A., Perroud, N., Aubry, J.-M., 2015. BDNF promoter I methylation correlates between post-mortem human peripheral and brain tissues. Neurosci. Res. 91, 1–7. https://doi.org/10.1016/j.neures.2014.10.003

197) Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.-N., Kubo, C., Koga, Y., 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J. Physiol. 558, 263–275. https://doi.org/10.1113/jphysiol.2004.063388

198) Suez, J., Zmora, N., Segal, E., Elinav, E., 2019. The pros, cons, and many unknowns of probiotics. Nat. Med. 25, 716–729. https://doi.org/10.1038/s41591-019-0439-x

199) Szafoni, S., Piegza, M., 2022. Progress in Personalized Psychiatric Therapy with the Example of Using Intranasal Oxytocin in PTSD Treatment. J. Pers. Med. 12, 1067. https://doi.org/10.3390/jpm12071067

200) Sze, Y., Brunton, P.J., 2024. How is prenatal stress transmitted from the mother to the fetus? J. Exp. Biol. 227, jeb246073. https://doi.org/10.1242/jeb.246073

201) Szyf, M., 2022. The epigenetics of early life adversity and trauma inheritance: an interview with Moshe Szyf. Epigenomics 14, 309–314. https://doi.org/10.2217/epi-2021-0483

202) Tan, S.Y., Yip, A., 2018. Hans Selye (1907–1982): Founder of the stress theory. Singapore Med. J. 59, 170–171. https://doi.org/10.11622/smedj.2018043

203) Tannock, G.W., 2004. A Special Fondness for Lactobacilli. Appl. Environ. Microbiol. 70, 3189–3194. https://doi.org/10.1128/AEM.70.6.3189-3194.2004

204) Teng, H.K., Teng, K.K., Lee, R., Wright, S., Tevar, S., Almeida, R.D., Kermani, P., Torkin, R., Chen, Z.-Y., Lee, F.S., Kraemer, R.T., Nykjaer, A., Hempstead, B.L., 2005. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J. Neurosci. Off. J. Soc. Neurosci. 25, 5455–5463. https://doi.org/10.1523/JNEUROSCI.5123-04.2005

205) Thul, T.A., Corwin, E.J., Carlson, N.S., Brennan, P.A., Young, L.J., 2020. Oxytocin and postpartum depression: A systematic review. Psychoneuroendocrinology 120, 104793. https://doi.org/10.1016/j.psyneuen.2020.104793

206) Toepfer, P., O'Donnell, K.J., Entringer, S., Garg, E., Heim, C.M., Lin, D.T.S., MacIsaac, J.L., Kobor, M.S., Meaney, M.J., Provençal, N., Binder, E.B., Wadhwa, P.D., Buss, C., 2019. Dynamic DNA methylation changes in the maternal oxytocin gene locus (OXT) during pregnancy predict postpartum maternal intrusiveness. Psychoneuroendocrinology 103, 156–162. https://doi.org/10.1016/j.psyneuen.2019.01.013

207) Tolkunova, K., Usoltsev, D., Moguchaia, E., Boyarinova, M., Kolesova, E., Erina, A., Voortman, T., Vasilyeva, E., Kostareva, A., Shlyakhto, E., Konradi, A., Rotar, O., Artomov, M., 2023. Transgenerational and intergenerational effects of early childhood famine exposure in the cohort of offspring of Leningrad Siege survivors. Sci. Rep. 13, 11188. https://doi.org/10.1038/s41598-023-37119-8

208) Tsankova, N., Renthal, W., Kumar, A., Nestler, E.J., 2007. Epigenetic regulation in psychiatric disorders. Nat. Rev. Neurosci. 8, 355–367. https://doi.org/10.1038/nrn2132

209) Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., Gordon, J.I., 2007. The Human Microbiome Project. Nature 449, 804–810. https://doi.org/10.1038/nature06244

210) Unternaehrer, E., Meyer, A.H., Burkhardt, S.C.A., Dempster, E., Staehli, S., Theill, N., Lieb, R., Meinlschmidt, G., 2015. Childhood maternal care is associated with DNA methylation of the genes for brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OXTR) in peripheral blood cells in adult men and women. Stress Amst. Neth. 18, 451–461. https://doi.org/10.3109/10253890.2015.1038992

211) Valtcheva, S., Issa, H.A., Bair-Marshall, C.J., Martin, K.A., Jung, K., Zhang, Y., Kwon, H.-B., Froemke, R.C., 2023. Neural circuitry for maternal oxytocin release induced by infant cries. Nature 621, 788–795. https://doi.org/10.1038/s41586-023-06540-4

212) van der Horst, F.C.P., Zetterqvist Nelson, K., van Rosmalen, L., van der Veer, R., 2020. A tale of four countries: How Bowlby used his trip through Europe to write the WHO report and spread his ideas. J. Hist. Behav. Sci. 56, 169–185. https://doi.org/10.1002/jhbs.22016

213) van Rosmalen, L., van der Veer, R., van der Horst, F.C., 2020. The nature of love: Harlow, Bowlby and Bettelheim on affectionless mothers. Hist. Psychiatry 31, 227–231. https://doi.org/10.1177/0957154X19898997

214) van Steenwyk, G., Roszkowski, M., Manuella, F., Franklin, T.B., Mansuy, I.M., 2018. Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation. Environ. Epigenetics 4.

https://doi.org/10.1093/eep/dvy023

215) Van Waes, V., Enache, M., Berton, O., Vinner, E., Lhermitte, M., Maccari, S., Darnaudéry, M., 2011. Effect of prenatal stress on alcohol preference and sensitivity to chronic alcohol exposure in male rats. Psychopharmacology (Berl.) 214, 197–208. https://doi.org/10.1007/s00213-009-1765-3

216) Vanbesien-Mailliot, C.C.A., Wolowczuk, I., Mairesse, J., Viltart, O., Delacre, M., Khalife, J., Chartier-Harlin, M.-C., Maccari, S., 2007. Prenatal stress has pro-inflammatory consequences on the immune system in adult rats. Psychoneuroendocrinology 32, 114–124. https://doi.org/10.1016/j.psyneuen.2006.11.005

217) Varian, B.J., Poutahidis, T., DiBenedictis, B.T., Levkovich, T., Ibrahim, Y., Didyk, E., Shikhman, L., Cheung, H.K., Hardas, A., Ricciardi, C.E., Kolandaivelu, K., Veenema, A.H., Alm, E.J., Erdman, S.E., 2017. Microbial lysate upregulates host oxytocin. Brain. Behav. Immun. 61, 36–49. https://doi.org/10.1016/j.bbi.2016.11.002

218) Verhaeghe, R., Gao, V., Morley-Fletcher, S., Bouwalerh, H., Van Camp, G., Cisani, F., Nicoletti, F., Maccari, S., 2021. Maternal stress programs a demasculinization of glutamatergic transmission in stress-related brain regions of aged rats. GeroScience 44, 1047–1069. https://doi.org/10.1007/s11357-021-00375-5

219) Waddington, C.H., 1942. The epigenotype. 1942. Int. J. Epidemiol. 41, 10–13. https://doi.org/10.1093/ije/dyr184

220) Wang, D., Y, L., Z, W., M, K., 2013. Histone deacetylase inhibitors facilitate partner preference formation in female prairie voles. Nat. Neurosci. 16. https://doi.org/10.1038/nn.3420

221) Wang, R., Wu, Z., Huang, C., Hashimoto, K., Yang, L., Yang, C., 2022. Deleterious effects of nervous system in the offspring following maternal SARS-CoV-2 infection during the COVID-19 pandemic. Transl. Psychiatry 12, 1–6. https://doi.org/10.1038/s41398-022-01985-z

222) Wang, T., Nakagawa, S., Miyake, T., Setsu, G., Kunisue, S., Goto, K., Hirasawa, A., Okamura, H., Yamaguchi, Y., Doi, M., 2020. Identification and functional characterisation of N-linked glycosylation of the orphan G protein-coupled receptor Gpr176. Sci. Rep. 10, 4429. https://doi.org/10.1038/s41598-020-61370-y

223) Wang, T., Shi, C., Li, X., Zhang, P., Liu, B., Wang, H., Wang, Y., Yang, Y., Wu, Y., Li, H., Xu, Z.-Q.D., 2018. Injection of oxytocin into paraventricular nucleus reverses depressive-like behaviors in the postpartum depression rat model. Behav. Brain Res. 336, 236–243. https://doi.org/10.1016/j.bbr.2017.09.012

224) Wastyk, H.C., Fragiadakis, G.K., Perelman, D., Dahan, D., Merrill, B.D., Yu, F.B., Topf,

M., Gonzalez, C.G., Treuren, W.V., Han, S., Robinson, J.L., Elias, J.E., Sonnenburg, E.D., Gardner, C.D., Sonnenburg, J.L., 2021. Gut-microbiota-targeted diets modulate human immune status. Cell 184, 4137-4153.e14. https://doi.org/10.1016/j.cell.2021.06.019

225) Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854. https://doi.org/10.1038/nn1276

226) Webb, E., Thomson, S., Nelson, A., White, C., Koren, G., Rieder, M., Van Uum, S., 2010. Assessing individual systemic stress through cortisol analysis of archaeological hair. J. Archaeol. Sci. 37, 807–812. https://doi.org/10.1016/j.jas.2009.11.010

227) Wei, M., Gao, Q., Liu, J., Yang, Y., Yang, J., Fan, J., Lv, S., Yang, S., 2023. Development programming: Stress during gestation alters offspring development in sheep. Reprod. Domest. Anim. Zuchthyg. 58, 1497–1511. https://doi.org/10.1111/rda.14465

228) Weickert, C.S., Hyde, T.M., Lipska, B.K., Herman, M.M., Weinberger, D.R., Kleinman, J.E., 2003. Reduced brain-derived neurotrophic factor in prefrontal cortex of patients with schizophrenia. Mol. Psychiatry 8, 592–610. https://doi.org/10.1038/sj.mp.4001308

229) Weinfeld, M., Sigal, J.J., Eaton, W.W., 1981. Long-Term Effects of the Holocaust on Selected Social Attitudes and Behaviors of Survivors: A Cautionary Note. Soc. Forces 60, 1–19. https://doi.org/10.2307/2577929

230) Weiss, I.C., Franklin, T.B., Vizi, S., Mansuy, I.M., 2011. Inheritable Effect of Unpredictable Maternal Separation on Behavioral Responses in Mice. Front. Behav. Neurosci. 5. https://doi.org/10.3389/fnbeh.2011.00003

231) Welberg, L.A.M., Seckl, J.R., Holmes, M.C., 2000. Inhibition of 11β-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. Eur. J. Neurosci. 12, 1047–1054. https://doi.org/10.1046/j.1460-9568.2000.00958.x

232) Windle, R.J., Shanks, N., Lightman, S.L., Ingram, C.D., 1997. Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. Endocrinology 138, 2829–2834. https://doi.org/10.1210/endo.138.7.5255

233) Winters, C., Gorssen, W., Ossorio-Salazar, V.A., Nilsson, S., Golden, S., D'Hooge, R., 2022. Automated procedure to assess pup retrieval in laboratory mice. Sci. Rep. 12, 1663. https://doi.org/10.1038/s41598-022-05641-w

234) Winters, C., Gorssen, W., Wöhr, M., D'Hooge, R., 2023. BAMBI: A new method for automated assessment of bidirectional early-life interaction between maternal behavior and pup vocalization in mouse dam-pup dyads. Front. Behav. Neurosci. 17, 1139254. https://doi.org/10.3389/fnbeh.2023.1139254

235) Wu, Y., De Asis-Cruz, J., Limperopoulos, C., 2024. Brain structural and functional outcomes in the offspring of women experiencing psychological distress during pregnancy. Mol. Psychiatry 1–18. https://doi.org/10.1038/s41380-024-02449-0

236) Xiong, R.-G., Li, J., Cheng, J., Zhou, D.-D., Wu, S.-X., Huang, S.-Y., Saimaiti, A., Yang, Z.-J., Gan, R.-Y., Li, H.-B., 2023. The Role of Gut Microbiota in Anxiety, Depression, and Other Mental Disorders as Well as the Protective Effects of Dietary Components. Nutrients 15, 3258. https://doi.org/10.3390/nu15143258

237) Yang, J., Harte-Hargrove, L.C., Siao, C.-J., Marinic, T., Clarke, R., Ma, Q., Jing, D., Lafrancois, J.J., Bath, K.G., Mark, W., Ballon, D., Lee, F.S., Scharfman, H.E., Hempstead, B.L., 2014. proBDNF negatively regulates neuronal remodeling, synaptic transmission, and synaptic plasticity in hippocampus. Cell Rep. 7, 796–806. https://doi.org/10.1016/j.celrep.2014.03.040

238) Yang, Y., Yu, H., Babygirija, R., Shi, B., Sun, W., Zheng, X., Zheng, J., 2019. Intranasal Administration of Oxytocin Attenuates Stress Responses Following Chronic Complicated Stress in Rats. J. Neurogastroenterol. Motil. 25, 611–622. https://doi.org/10.5056/jnm19065

239) Yang, Z., Li, J., Gui, X., Shi, X., Bao, Z., Han, H., Li, M.D., 2020. Updated review of research on the gut microbiota and their relation to depression in animals and human beings. Mol. Psychiatry 25, 2759–2772. https://doi.org/10.1038/s41380-020-0729-1

240) Yao, S., Kendrick, K.M., 2022. Effects of Intranasal Administration of Oxytocin and Vasopressin on Social Cognition and Potential Routes and Mechanisms of Action. Pharmaceutics 14, 323. https://doi.org/10.3390/pharmaceutics14020323

241) Yehuda, R., Bell, A., Bierer, L.M., Schmeidler, J., 2008. Maternal, not paternal, PTSD is related to increased risk for PTSD in offspring of Holocaust survivors. J. Psychiatr. Res. 42, 1104–1111. https://doi.org/10.1016/j.jpsychires.2008.01.002

242) Yehuda, R., Bierer, L.M., 2007. Transgenerational transmission of cortisol and PTSD risk, in: De Kloet, E.R., Oitzl, M.S., Vermetten, E. (Eds.), Progress in Brain Research, Stress Hormones and Post Traumatic Stress Disorder Basic Studies and Clinical Perspectives. Elsevier, pp. 121–135. https://doi.org/10.1016/S0079-6123(07)67009-5

243) Yehuda, R., Daskalakis, N.P., Bierer, L.M., Bader, H.N., Klengel, T., Holsboer, F., Binder, E.B., 2016. Holocaust Exposure Induced Intergenerational Effects on FKBP5 Methylation. Biol. Psychiatry 80, 372–380. https://doi.org/10.1016/j.biopsych.2015.08.005

244) Yehuda, R., Daskalakis, N.P., Lehrner, A., Desarnaud, F., Bader, H.N., Makotkine, I., Flory, J.D., Bierer, L.M., Meaney, M.J., 2014. Influences of maternal and paternal PTSD on epigenetic regulation of the glucocorticoid receptor gene in Holocaust survivor offspring. Am. J. Psychiatry 171, 872–880. https://doi.org/10.1176/appi.ajp.2014.13121571

245) Yehuda, R., Engel, S.M., Brand, S.R., Seckl, J., Marcus, S.M., Berkowitz, G.S., 2005. Transgenerational Effects of Posttraumatic Stress Disorder in Babies of Mothers Exposed to the World Trade Center Attacks during Pregnancy. J. Clin. Endocrinol. Metab. 90, 4115–4118. https://doi.org/10.1210/jc.2005-0550

246) Yehuda, R., Halligan, S.L., Grossman, R., 2001. Childhood trauma and risk for PTSD: relationship to intergenerational effects of trauma, parental PTSD, and cortisol excretion. Dev. Psychopathol. 13, 733–753. https://doi.org/10.1017/s0954579401003170

247) Yehuda, R., Morris, A., Labinsky, E., Zemelman, S., Schmeidler, J., 2007. Ten-year follow-up study of cortisol levels in aging holocaust survivors with and without PTSD. J. Trauma. Stress 20, 757–761. https://doi.org/10.1002/jts.20228

248) Yehuda, R., Schmeidler, J., Labinsky, E., Bell, A., Morris, A., Zemelman, S., Grossman, R.A., 2009. Ten-Year Follow-Up Study of PTSD Diagnosis, Symptom Severity, and Psychosocial Indices in Aging Holocaust Survivors. Acta Psychiatr. Scand. 119, 25–34. https://doi.org/10.1111/j.1600-0447.2008.01248.x

249) Yonkers, K.A., Smith, M.V., Forray, A., Epperson, C.N., Costello, D., Lin, H., Belanger, K., 2014. Pregnant women with posttraumatic stress disorder and risk of preterm birth. JAMA Psychiatry 71, 897–904. https://doi.org/10.1001/jamapsychiatry.2014.558

250) Zhang, T.Y., Labonté, B., Wen, X.L., Turecki, G., Meaney, M.J., 2013. Epigenetic Mechanisms for the Early Environmental Regulation of Hippocampal Glucocorticoid Receptor Gene Expression in Rodents and Humans. Neuropsychopharmacology 38, 111–123. https://doi.org/10.1038/npp.2012.149

251) Zhao, Y.-F., Wei, D.-N., Tang, Y., 2021. Gut Microbiota Regulate Astrocytic Functions in the Brain: Possible Therapeutic Consequences. Curr. Neuropharmacol. 19, 1354–1366. https://doi.org/10.2174/1570159X19666210215123239

252) Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. Int. J. Syst. Evol. Microbiol. 70, 2782–2858. https://doi.org/10.1099/ijsem.0.004107

253) Zinni, M., Colella, M., Batista Novais, A.R., Baud, O., Mairesse, J., 2018. Modulating the Oxytocin System During the Perinatal Period: A New Strategy for Neuroprotection of the Immature Brain? Front. Neurol. 9.

254) Ziomkiewicz, A., Babiszewska, M., Apanasewicz, A., Piosek, M., Wychowaniec, P., Cierniak, A., Barbarska, O., Szołtysik, M., Danel, D., Wichary, S., 2021. Psychosocial stress

and cortisol stress reactivity predict breast milk composition. Sci. Rep. 11, 11576. https://doi.org/10.1038/s41598-021-90980-3

255) Zoubovsky, S.P., Hoseus, S., Tumukuntala, S., Schulkin, J.O., Williams, M.T., Vorhees, C.V., Muglia, L.J., 2020. Chronic psychosocial stress during pregnancy affects maternal behavior and neuroendocrine function and modulates hypothalamic CRH and nuclear steroid receptor expression. Transl. Psychiatry 10, 1–13. https://doi.org/10.1038/s41398-020-0704-2

256) Zuena, A.R., Mairesse, J., Casolini, P., Cinque, C., Alemà, G.S., Morley-Fletcher, S., Chiodi, V., Spagnoli, L.G., Gradini, R., Catalani, A., Nicoletti, F., Maccari, S., 2008. Prenatal Restraint Stress Generates Two Distinct Behavioral and Neurochemical Profiles in Male and Female Rats. PLoS ONE 3. https://doi.org/10.1371/journal.pone.0002170